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(54) Title: ANALYSIS OF ALPHA INTEGRINS FOR THE DIAGNOSIS OF DIABETIC NEPHROPATHY

(57) Abstract

Analysis of alterations in integrin subunit expression, particularly  $\alpha 1$  and/or  $\alpha 2$  integrin subunit expression from integrin producing cells as compared to normal controls as a diagnostic method to identify individuals who have or are predisposed to pathologies associated with altered matrix deposition, such as diabetic renal nephropathy.

*immunofluorescence*  
C p 9 13  
*immunoassay*  
C p 16  
C p 66

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## ANALYSIS OF ALPHA INTEGRINS FOR THE DIAGNOSIS OF DIABETIC NEPHROPATHY

### Background of the Invention

5        Diabetic nephropathy is a major cause of renal failure in the U.S. and develops in approximately 30% of insulin dependent diabetes mellitus (IDDM) patients. Recent studies by the Diabetes Control and Complications Trial Group have indicated that intensive insulin treatment substantially reduces the risk of developing complications, including nephropathy. However, the cost and effort of the intensive therapy, as well as  
10      the danger of hypoglycemic attacks dictate that this treatment should be limited to those patients who are prone to develop complications. It follows that an early selection of these diabetic subjects would be extremely helpful, but currently there are no adequate predictors available for clinical use.

15      Metabolic imbalance caused by hyperglycemia has been implicated as a major factor in the development of this condition and is associated with a genetic tendency to develop nephropathy. A prominent expansion of the mesangium with changes in the composition of the mesangial matrix have been observed in diabetic nephropathy (Williamson et al., *Diabetes Met. Rev.* 4:339 (1988), Steffes, M.W., et al. *Diabetes* 38:1077-81 (1989)).

20      Studies performed with human and experimental animal mesangial cells cultured in high-glucose medium have demonstrated an increased synthesis and accumulation of matrix proteins, namely collagens, including collagen type IV and fibronectin. This suggests that hyperglycemia plays a role in the mesangial changes of diabetic nephropathy. Ayo, S.H., et al. (1990a), *Am. J. Pathol.* 136:1339-1348; Nahman, N.S., et al., *Kidney Int.* 41:396-402 (1992); Danne, T., et al., *Diabetes* 42:170-177 (1993). The changes in the matrix secretion pattern of the cell are mediated either directly by hyperglycemia or by the glycation of mesangial matrix on prolonged exposure to high levels of glucose. Studies have demonstrated that cultured mesangial cells are influenced by the glycation of matrix leading to altered cell adhesion, spreading and proliferation. Since collagen IV (cIV) is the major component of the mesangial matrix (about 60%), changes in the interactions between this major mesangial glycoprotein and mesangial cells may play an important role in the pathology of diabetic nephropathy. Kim, Y., et al., *Am. J. Pathol.* 138:413-420 (1991). The changes in matrix deposition

are secondary in time to insulin insufficiency. Altered matrix deposition including basement membrane thickening is also found in a variety of arterioles and arteries in patients with diabetes mellitus. Altered matrix deposition is found in the pancreas of diabetic patients. Altered matrix deposition puts diabetic patients at risk for developing secondary pathological changes including, but not limited to nephropathy, myocardial infarction, cerebral stroke, problems associated with reduced circulation, retinopathy, neuropathies and the like.

Cell-matrix interactions are mediated, for the most part, by a family of receptors known as integrins. The very late antigen (VLA) subgroup of integrins which share a common  $\beta 1$  chain, include the cell membrane receptors for cIV,  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$ .

Although integrins are mainly studied for their role in cell differentiation, migration and signaling events, they may also be involved in the maintenance of tissue structure. For instance, cells can modify their matrix by altering the production of matrix proteins and/or by regulating matrix organization. Cells cultured under high glucose conditions resulted in an increased production of matrix components by mesangial cells.

(Kashgarian, M., et al., *Kidney Int.* 41:524-529 (1992).) The balance of cell surface integrin expression has been demonstrated to be altered in various disease states including inflammation and malignancy (Waes and Carey, *Otolaryngologic Clinics of North America* 25(5):1117 (1992); Adams, J.C., et al., *Cell* 63:425-435 (1990); Rozzo et al., *FEBS Letters* 332:263 (1993)). This altered expression has been associated with altered adhesion to extracellular components.

Presently, the only earliest available indicator of kidney changes is microalbuminuria which occurs after the appearance of nephropathic changes. Yet only a percentage of individuals with microalbuminuria go on to develop glomerulopathy.

Individuals at risk for developing glomerulopathy are best treated with intense glucose-modulating therapies that have their own risk. Often physicians are hesitant to place individuals with microalbuminuria on such therapies since the majority of these patients do not proceed to glomerulopathy. Biopsies indicating the accumulation of matrix accompanying the expansion of the mesangium occur at a point when the process has become irreversible. Therefore an early predictor of nephropathy or other disease states associated with altered matrix deposition would be beneficial as an indicator of those

patients who require stringent control of blood glucose levels to minimize nephropathic and other altered matrix deposition-associated disorders.

Thus, there is a need to identify markers associated with the changes seen in nephropathy and in other altered matrix deposition-associated disorders for the diagnosis of these disorders. There is a need to identify changes in regulation and function of integrins in diabetic patients and there is a need to develop a diagnostic test that can be used to identify patients who are likely to develop or have the early symptoms of nephropathy.

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#### Summary of the Invention

Alterations in the amounts and patterns of alpha-integrin subunits has now been correlated to the onset of nephropathy. Analysis of alpha integrin subunit expression as compared with controls provides a diagnostic tool for the determination of patients likely to develop severe nephropathy and a method to monitor progress of disease during treatment protocols.

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Cells that express alpha integrins, such as kidney tissue, fibroblasts, endothelial cells, and blood cells are analyzed for alpha integrin subunit expression, for example, by *in situ* hybridization methods. Changes in the amounts and pattern of integrin subunit expression as compared with control samples, is diagnostic of nephropathy and can be used to screen individuals, e.g., diabetic patients at risk for developing severe disease.

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Analysis of  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ , and beta-1 integrin subunit expression as compared with control tissue expression is preferred. An increase in  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ , or beta-1 integrin expression and/or a decrease in  $\alpha 1$  expression is diagnostic of increased risk of nephropathy. An especially preferred diagnostic method is the comparison of  $\alpha 1$  and  $\alpha 2$  integrin subunit expression with control tissue. A pattern change including a decrease in  $\alpha 1$  and an increase in  $\alpha 2$  is diagnostic of increased risk of nephropathy or onset of the disease.

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#### Brief Description of the Drawings

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Figure 1 is a histogram summarizing results of *In situ* hybridization studies of rat control and diabetic tissue with  $\alpha 1$  and  $\alpha 2$  integrin probes.

### Detailed Description of the Invention

Analysis of changes in the pattern of integrin subunit expression, particularly of alpha integrin subunits, is made by comparing expression in sample tissues as compared with tissue controls.

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#### **Tissue Samples:**

The invention is directed to methods of detecting changes in  $\alpha$  integrin subunit expression in cells, such as the cell populations (visceral epithelial, endothelial and mesangial and other matrix-producing cells) present in the glomerulus; and also in the tubules as well as including, but not limited to, fibroblasts (for example see D. Kyu Jin, et al. in *J. Am. Society of Nephrology*, 5(3): 966, 1994), epithelial, and endothelial cells from a variety of tissues and organs as well as blood cells including, but not limited to polymorphonuclear leukocytes, monocytes, and the like. Changes to blood cells, including leukocytes, have been reported in diabetic patients who develop nephropathy (Ng, et al. *Diabetologia* 33:278-284, 1990).

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A change in the expression of  $\alpha 1$  and  $\alpha 2$  integrins has been detected in the studies disclosed here, under conditions of high glucose (i.e., about 25 mM) compared with low glucose (i.e., about 5 mM), in diabetic test animals *in vitro*, and in a human diabetic patient with neuropathy. Mesangial cells cultured in high glucose showed an increase in  $\alpha 2$  integrin expression and a decrease in  $\alpha 1$  integrin expression compared with mesangial cells grown under low glucose conditions. A change in expression of  $\alpha$  integrins such as  $\alpha 1$  and/or  $\alpha 2$  subunits can be used to identify patients that have or will develop diabetic nephropathy. In view of these studies, it is believed that patients showing about a 25 to 100% decrease in  $\alpha 1$  integrin and/or about a 25 to 100% increase in  $\alpha 2$  integrin expression have a greater chance of developing diabetic nephropathy. The methods disclosed here are useful to identify diabetic patients at risk for developing diabetic nephropathy. The methods may also be useful to monitor progression of diabetic nephropathy. Patients identified as having a risk for developing or showing early symptoms of diabetic nephropathy can be placed on a strict glucose control regimen so that the development and/or progression of nephropathy can be inhibited.

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Changes in integrin subunit expression in diabetic patients have been identified in cultured human skin fibroblasts taken from skin biopsies (D. Kyu Jin, et al., *J. Am.*

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*Soc. of Nephrology* 5(3):966, 1994) suggesting that a variety of integrin-expressing cells could be monitored to identify individuals with a predisposition to nephropathy or to other complications associated with diabetes-induced altered matrix deposition.

5      **Methods of Detecting a Change in Expression of  $\alpha$ .1 and/or  $\alpha$ .2 Integrin Subunits in Cells from Diabetic Patients**

The methods of the invention are conducted with cell types that express alpha ( $\alpha$ ) integrin subunits. Preferably, to identify patients predisposed to nephropathy, the cells are obtained from tissue samples from biopsy of kidney tissue of diabetic patients. 10      However, other cell types that express  $\alpha$  integrin subunits can be utilized including, but not limited to, fibroblasts, endothelial cells, polymorphonuclear leukocytes, monocytes, and other blood cells. The amount of cells typically obtained is relatively small so that the detection methods selected are those that can detect and/or quantitate  $\alpha$  integrin subunit expression in a small cell sample. These methods include, but are not limited to 15      *in situ* hybridization, including polymerase chain reaction (PCR) enhanced *in situ* hybridization (also known as *in situ* PCR) and the like.

The cell samples are obtained from patients having diabetes but having no demonstrable symptoms or signs of nephropathy. The earliest change in nephropathy is the detection of microalbuminuria. Biopsy specimens may also be obtained from 20      diabetic patients that may have early symptoms of nephropathy so that the progression of diabetic nephropathy can be monitored. Blood samples and skin biopsies also can be obtained from patients with diabetes and processed for either *in situ* hybridization or PCR enhanced *in situ* hybridization (also known as *in situ* PCR). Similarly, it is possible to perform *in situ* hybridization or PCR enhanced *in situ* hybridization using a 25      cheek scraping or a scraping of other accessible tissue.

Biopsy tissue samples are usually about 1mm<sup>3</sup> and are obtained using standard biopsy methods. Where the kidney is the organ selected for biopsy, kidney tissue from the cortical region is preferred although biopsy samples can be obtained elsewhere. Fibroblasts can be obtained from skin or any other tissue. The biopsy samples are then 30      frozen in liquid nitrogen or fixed in 4% fresh paraformaldehyde and sectioned into 5  $\mu$ m thick sections on silane-coated slides. The sections can then be treated with reagents to detect and/or quantitate  $\alpha$  integrin expression in cells.

Blood cells and other  $\alpha$  integrin expressing cells can also be analyzed for changes in  $\alpha$  integrin subunit expression. These cells include fibroblasts, monocytes, polymorphonuclear leukocytes and other blood cells. Cells can be obtained and isolated from a blood or bone marrow sample. Methods for isolating particular cell types from a 5 blood sample are well known in the art. Preferably leukocytes are isolated from blood by centrifugation, followed by hypotonic shock of residual blood cells as disclosed by Ng, et al. *Diabetologia* 33:278-284, 1990.

Rather than preparing cell sections, the sample of cells can be extracted to obtain 10 nucleic acids using standard methods. The nucleic acids encoding  $\alpha 1$  and/or  $\alpha 2$  integrin subunits can be amplified using any of a variety of polymerase chain reaction methods. For example, changes in the level of expression of  $\alpha 1$  and/or  $\alpha 2$  integrins can be detected using a competitive PCR method as described by Gilland, G., *Proc. Natl. Acad. Sci. (USA)* 87:2725 (1990).

In a method of the invention, the level of  $\alpha 1$  integrin expression is detected 15 and/or quantitated in cells such as glomerular and tubular kidney cells. The level of  $\alpha 1$  integrin expression can be detected using a variety of standard methods. The preferred methods are *in situ* hybridization, *in situ* PCR for detection of integrin RNA and immunofluorescence detection of antibody-tagged integrin protein. A decrease of about 20 25 to 100% in  $\alpha 1$  integrin expression can indicate that early changes of diabetic nephropathy are occurring and can be used to identify patients that have an increased risk of developing diabetic nephropathy. A decrease in  $\alpha 1$  integrin expression is compared to the level of  $\alpha 1$  integrin expression in cells from age matched non-diabetic controls.

For detection and quantitation using *in situ* hybridization, the following method 25 is preferred: a detectably labeled probe that is complementary to and/or hybridizes to all or a portion of nucleic acid sequences encoding all or a portion of  $\alpha 1$  integrin subunit is utilized. A radioactively labeled probe preferably has a specific activity of about  $2 \times 10^8$  to  $1 \times 10^9$  dpm/ $\mu$ g. *In situ* hybridization on cells such as kidney tissue can be conducted as follows. 5  $\mu$ m tissue sections, fibroblasts and/or blood cells on silane-coated slides 30 are further fixed in fresh 4% paraformaldehyde for 10 min. The slides are then pretreated with 0.2N HCl for 20 min., 0.05 M Triethanolamine (TEA, Sigma) for 15 min, 0.005% digitonin for 5 min., 3  $\mu$ g/ml proteinase K (Sigma) for 15 min. at 37°C,

and 0.3% acetic anhydride - 0.1M TEA for 10 min. Hybridization is performed at 50°C overnight in 50% formamide, 0.6 M NaCl, 1xDenhardt's, 0.17 µg/ml human COT<sup>RT</sup> DNA (GIBCO/BRL), 1 mg/ml poly A (Boehringer Mannheim), 10% (W/V) Dextran sulfate (Sigma), 0.1 M dithiothreitol (DTT, Boehringer Mannheim), 1 mM EDTA, 0.1 mM aurinitricarboxylic acid (ATA, Sigma) and S<sup>35</sup>-dCTP labeled cDNA probe. The following day, the slides are washed in 2x SCC-0.05% SDS for 60 min. at 55°C; further washed in the high stringency washing buffer containing 50% formamide, 0.6 M NaCl, 1 mM EDTA, 5 mM DTT and 10 mM Hepes for 4 days at room temperature. After 4 days, the slides are rinsed in 2x SCC and the slides are dehydrated in graded ethanol with 0.3 M ammonium acetate, then dipped in Kodak NTB-2 emulsion and exposed for 5 days at 4°C. After development, the slides are stained with hematoxylin-eosin (Surgipath Canada, Inc., Winnipeg, Canada) and mounted. The silver grain number per cell are used to quantitate the result of *in situ* hybridization. About 10-20 glomeruli and a similar number of tubules are examined per patient.

A probe of the invention hybridizes to and is complementary to and/or all or a portion of a nucleic acid sequence encoding  $\alpha 1$  integrin as long as the probe specifically detects  $\alpha 1$  integrin expression. Probes can be designed using a known sequence such as the rat  $\alpha 1$  integrin sequence as shown as Figure 2 in Takada and Hemnlev, *J. Cell Biol.* 109:397-407 (1983) or by the use of commercially available programs and are capable of binding to rodent or human  $\alpha 1$  integrin but are not capable of binding to other proteins including other proteins having regions homologous to  $\alpha$  integrins when tested under identical hybridization conditions. Examples of other proteins that have homologous regions to  $\alpha$  integrins include those proteins identified using a gene bank search, such as GenBank, or the like, or in publications related to  $\alpha 1$  and  $\alpha 2$  subunits (for example, see Ignatius, et al. *J. Cell Biol.* 111:709-720, 1990 listing proteins with homologies to the  $\alpha 1$ -subunit).

The probe can be about 15 nucleotides long up to a full length probe of about 4kb. The probes are preferably 100% complementary to the nucleic acid encoding  $\alpha 1$  integrin however some mismatches can be present depending on the length of the probe. About 1 to 3 mismatches in a probe of about 20 to 30 nucleotides long can be present as long as hybridization conditions are adjusted to account for mismatches. Hybridization conditions can be adjusted to take into account mismatches in accord with known

principles as described in Sambrook et al., A Guide to Molecular Cloning, Cold Spring Harbor NY (1989).

A specific example of a nucleic acid sequence encoding  $\alpha 1$  integrin is the rat  $\alpha 1$  integrin sequence shown as Figure 2 in Ignatius et al., *J. Cell. Biol.* 111:709-720, 1990, 5 (SEQ ID NO:1) and the protein sequence encoded by  $\alpha 1$  integrin is provided as SEQ ID NO:2. A DNA sequence encoding  $\alpha 1$  integrin can be obtained from a rat pheochromocytoma cell line PC12 as described by Ignatius et al., *J. Cell. Biol.* 111:709 (1990). Briefly, a cDNA library can be prepared from rat pheochromocytoma PC12 in a lambda vector. The sequence can be identified and/or amplified using probes or 10 primers designed from the known sequences using standard methods as described in Sambrook et al., (*supra*). Once the sequence is subcloned it can be confirmed by sequence analysis and/or by screening with antibodies specific for  $\alpha 1$  integrin. Other DNA sequences encoding  $\alpha 1$  integrins can be identified and isolated using probes and primers derived from the known sequences.

15 A preferred probe is a 3.9 kb fragment from the 5' end through the EcoR1 site near base 3900 including the sequence as shown in Figure 2 of Ignatius et al. (*supra*). Smaller fragments that can form probes can readily be prepared with restriction enzymes or derived by automated or manual oligonucleotide synthesis techniques, by PCR, or by other methods also known in the art. The probes are preferably detectably 20 labeled with a radioactive nucleotide using standard methods.

Other methods utilizing probes for detection of  $\alpha 1$  integrin expression can also be utilized using standard methods such as Northern Blot Analysis and the like as described in Sambrook et al., cited *supra*.

Primers can also be designed based upon the sequence of rat  $\alpha 1$  integrin 25 sequence. This invention also contemplates using primers and nucleic acid sequences from the human  $\alpha 1$  integrin sequence provided by Briesewitz, et al. (*J. Biol. Chem.* 268(4):2989-96, 1993). Primers can be designed using a known sequence using commercially available computer programs. Primers typically are complementary to and/or hybridize to a 5' region and/or a 3' region of the nucleic acid sequence encoding 30 the protein of interest. The primers can be used to amplify all or a portion of DNA or cDNA encoding  $\alpha 1$  integrin. Primers can be used to make probes and to detect expression levels of  $\alpha 1$  integrin. Primers preferably have at least 15 nucleotides that are

100% complementary to the nucleotide sequence selected. The primers can also have additional sequences preferably at the ends of the primer that include restriction enzyme sites and the like that are not complementary to the nucleic acid sequence to be amplified. Primers are preferably about 15 to 50 nucleotides long and can be prepared by automated synthesis.

5           The primers can be used to detect the level of  $\alpha 1$  expression in cells. RNA from cells is extracted and reverse transcribed using standard methods. Primers that are complementary to and can hybridize to a DNA sequence encoding  $\alpha 1$  integrins are utilized to amplify the cDNA. A decrease in the level of PCR product can be determined in comparison to the amount of PCR product obtained from control cells.

10           One method of utilizing PCR to detect  $\alpha 1$  integrin expression is *in situ* PCR. A method for PCR *in situ* hybridization is described in PCR In Situ Hybridization Protocols and Applications, J. Novo ed., "PCR *In Situ* Hybridization", pp. 157-183. Briefly, tissue sections, fibroblasts and/or blood cells (about 5  $\mu\text{m}$ ) are placed on silane-coated glass slides. After removing paraffin, the slides are treated with trypsinogen (2mg/ml) in 0.01N HCl for 10 minutes and then trypsinogen inactivated in 0.1M Tris HCl (pH 7.0) solution. The slides are washed sequentially in 90% and 100% ethanol, two times for 1 minute each and air dried. Aliquots of reaction mixture containing 0.15 units/ml *Taq* DNA polymerase and specific primer pairs for  $\alpha 1$  integrin are added to the 15 tissue section and then overlaid with siliconized glass coverslips. The slides are placed in the heat-sealable plastic bags and 4-5ml mineral oil is added. After removing air, the bag is heat-sealed and placed in the thermal-cycling oven for 40 cycles. After thermal-cycling, the slides are washed twice in chloroform for 2 minutes. The coverslips are removed and the slides are dipped briefly in fresh chloroform. After washing in PBS for 20 5 minutes, the slides are dehydrated and air-dried. The slides are dipped in NTB2 nuclear emulsion (Kodak) and exposed in the dark for 7 days. After development, the 25 slides are *counterstained* with hematoxylin-eosin.

30           A change in the level of  $\alpha 1$  integrin protein expression can also be detected by using immunofluorescence. (Unless otherwise specified as "protein expression", the term "expression" used herein generally refers to RNA expression.) Sections of tissue samples, fibroblasts and/or blood cells can be stained with antibodies specific for  $\alpha 1$  integrin. It is preferable that antibodies are monoclonal antibodies and are antibodies

that do not substantially cross-react with other  $\alpha$  integrin subunits. Antibodies to  $\alpha 1$  integrin can be made by standard methods such as described in Wayner EA and WG Carter, 1987, *J. Cell Biol.* 121(5):1141-1152. Antibodies specific to  $\alpha 1$  integrin include the SR84 and TS2/7 antibodies. Information related to these antibodies is provided in Examples 1 and 3. A decrease in the level of immunofluorescence can be observed and quantitated using standard methods. A decrease of about 25 to 100% of  $\alpha 1$  integrin expression may be used to identify patients that have a greater risk of developing diabetic nephropathy. A decrease in  $\alpha 1$  integrin expression is compared to the level of  $\alpha 1$  integrin expression in age-matched nondiabetic controls.

10       The preferred method of the invention involves comparing the level of expression of  $\alpha 2$  integrin to the level of expression of  $\alpha 1$  integrin. Under high glucose conditions, a decrease in the level of  $\alpha 1$  expression is seen as well as an increase in the level of  $\alpha 2$  expression in mesangial cells. It is believed that patients at greater risk for nephropathy or other complications associated with diabetes will exhibit an increase in  $\alpha 2$  expression and a decrease in  $\alpha 1$  expression. A change of about 15 to 100%, and preferably of about 25 to 100%, of  $\alpha 2$  integrin expression as well as a change of about 15 to 100%, and preferably of about 25 to 100%, of  $\alpha 1$  integrin expression is believed to be indicative of patients with a greater risk of developing diabetic nephropathy.

15       Integrin expression is associated with a variety of cell types in a variety of locations throughout the body, therefore it is possible that altered levels of integrin expression will also be identified in diabetic associated retinopathy, atherosclerosis and select diabetic neuropathies.

20       The expression of integrin subunits, preferably of  $\alpha 1$  and  $\alpha 2$  integrin subunits, is detected and/or quantitated in tissue samples, fibroblasts and/or blood cells from diabetic patients. The preferred methods are those that allow detection of gene expression in a small amount of cells or tissue.

25       The expression of  $\alpha 2$  integrin can be detected using *in situ* hybridization. The conditions for *in situ* hybridization are the same as those described previously. A probe specific for nucleic acid sequences encoding  $\alpha 2$  integrin can be prepared using standard methods as described in Sambrook et al., cited *supra*. The probes are complementary to and/or hybridize to all or a portion of a nucleic acid sequence encoding  $\alpha 2$  integrin. As described for  $\alpha 1$  integrin, the probe to detect  $\alpha 2$  integrin can hybridize to a portion of a

nucleic acid sequence as long as the probe specifically detects a sequence encoding  $\alpha 2$  integrin. Nucleic acid sequences can be DNA, cDNA, or RNA. It is preferred that the probe hybridize to RNA or cDNA.

A specific example of nucleic acid sequence encoding  $\alpha 2$  integrin is shown in 5 Figure 2 of Takada and Hemler, *J. Cell Biol.* 109:397 (1989). (SEQ ID NO:3). DNA sequence encoding human  $\alpha 2$  integrin can be isolated as described in this reference. The protein encoded by SEQ ID NO:3 is provided in this disclosure as SEQ ID NO:4. Nucleic acid sequences encoding  $\alpha 2$  integrin can be obtained from human lung 10 fibroblasts and/or human endothelial cells. Preferably DNA libraries from endothelial cells can be prepared and nucleic acids encoding  $\alpha 2$  integrin identified and/or amplified using probes and primers derived from the sequence of  $\alpha 2$  integrin, e.g., as shown in Figure 2 of Takada et al. (*supra*). If primers are selected, DNA sequences can be 15 amplified using the polymerase chain reaction and then subcloned. Clones that are positive by hybridization to a probe specific for DNA sequences encoding  $\alpha 2$  integrin (see Examples 1 and 3) or that express proteins that are positive by reacting with an antibody specific to  $\alpha 2$  integrin such as P1H5 are selected. A DNA sequence encoding  $\alpha 2$  integrin can be confirmed by DNA sequencing in comparison to the known  $\alpha 2$  sequence, as shown in Figure 2 of Takada et al. (*supra*).

A probe of the invention hybridizes to and is complementary to and/or 20 hybridizes to all or a portion of a nucleic acid sequence encoding  $\alpha 2$  integrin as long as the probe specifically detects  $\alpha 2$  integrin expression. Probes can be designed using a known sequence such as shown in Figure 2 of Takada et al. (*supra*) by the use of commercially available programs.

The probe can be about 15 nucleotides long up to a full length probe of about 25 5Kb. The probes are preferably 100% complementary to the nucleic acid encoding  $\alpha 2$  integrin however some mismatches can be present depending on the length of the probe. About 1 to 3 mismatches in a probe of about 20 to 30 nucleotides long can be present as long as hybridization conditions are adjusted to account for mismatches. Hybridization conditions can be adjusted to take into account mismatches in accord with known 30 principles are described in Sambrook et al., A Guide to Molecular Cloning, Cold Spring Harbor NY (1989).

A preferred probe is a 1.8 fragment kb from the 5' end through the EcoR1 site near base 1800 of the sequence shown in Figure 2 of Takada et al. (*supra*). Other probes can be derived from this fragment or from the full length sequence by use of restriction enzyme digestion. Probes can also be prepared by automated synthesis or by 5 PCR. Probes are preferably detectably labeled with a radioactive nucleotide using standard methods.

- Probes specific for  $\alpha 2$  integrin expression can then be utilized in methods of detecting  $\alpha 2$  integrin expression in various cell types. The preferred method is by use of *in situ* hybridization or PCR-*in situ* hybridization on kidney as well as other tissues.
- 10 The method utilized for *in situ* hybridization has been described previously (Takada and Hemler, *supra*). The method for PCR *in situ* hybridization has been described for  $\alpha 1$  integrin. Other methods utilizing probes for detection of  $\alpha 2$  integrin expression can also be utilized using standard methods such as Northern Blot Analysis, and the like, as described in Sambrook et al. cited *supra*.
- 15 Primers can also be designed based upon the known DNA sequence encoding human  $\alpha 2$  integrin. Primers can be designed from a known sequence such as shown in Figure 2 of Takada et al. (*supra*), using commercially available software. Primers typically are complementary to and/or hybridize to a 5' region and/or a 3' region. The primers can be used to amplify all or a portion of DNA or cDNA encoding  $\alpha 2$  integrin.
- 20 Primers can be used to make probes and to detect expression levels of  $\alpha 2$  integrin. Primers preferably have at least 15 nucleotides that are 100% complementary to the nucleotide sequence selected. The primers can also have additional sequence preferably at the ends of the primer that include restriction enzyme recognition sites and the like. Primers are preferably about 15 to 50 nucleotides long and can be prepared by 25 automated synthesis.
- Primers can be used to detect the level of  $\alpha 2$  integrin expression in cells. Nucleic acids, preferably RNA, from cells from diabetic patients are extracted and reverse transcribed using a standard method. Primers that are complementary to and can hybridize to a cDNA sequence encoding  $\alpha 2$  integrin are utilized to amplify the cDNA.
- 30 An increase in the level of PCR product can be determined in comparison to the amount of PCR product obtained from control cells.

A change in the level of  $\alpha 2$  integrin protein expression can also be detected by using immunofluorescence. Sections from kidneys and/or other tissues, skin fibroblasts and/or blood cells can be incubated with antibodies specific to  $\alpha 2$  integrin. It is preferable that the antibodies are monoclonal antibodies and are antibodies that do not crossreact with other  $\alpha$  integrin subunits. Antibodies to  $\alpha 2$  integrin can be made by standard methods such as described in Wayner EA and WG Carter, 1987, *J. Cell Biol.* 121(5):1141-1152. Antibodies specific for  $\alpha 2$  integrin include P1H5. An increase in the level of immunofluorescence can be observed and quantitated using standard methods such as flow cytometry. An increase of about 25 to 100% of  $\alpha 2$  integrin expression can be used to identify patients that have a greater risk of developing diabetic nephropathy. An increase in  $\alpha 2$  integrin expression is compared to  $\alpha 2$  integrin expression in nondiabetic control cells.

An increase in  $\alpha 2$  integrin expression alone can also be used to identify a patient that may have a greater risk of developing diabetic nephropathy. An increase in  $\alpha 2$  expression can be determined as described using the methods described above. An increase of about 25 to 100% in  $\alpha 2$  integrin expression may indicate a patient who has an increased risk of developing diabetic nephropathy.

Although an increase of  $\alpha 2$  integrin expression or a decrease of  $\alpha 1$  integrin expression alone can be utilized to identify patients at greater risk for developing diabetic nephropathy, a preferred method is to detect changes in both  $\alpha 1$  and  $\alpha 2$  integrin expression. It is believed that an increase in  $\alpha 2$  integrin expression and a decrease in  $\alpha 1$  integrin expression identifies patients that are at greater risk of or are showing early symptoms of diabetic nephropathy.

In one step of the method, the level of  $\alpha 2$  to  $\alpha 1$  integrin is compared. The level of  $\alpha 1$  integrin expression can be detected and/or quantitated using the methods described previously. The level of  $\alpha 1$  and  $\alpha 2$  integrin expression can be quantitated on two different cell samples such as two sections of the same tissue sample. About 10-20 glomeruli and tubules are examined. On one cell sample containing the same type of cells,  $\alpha 2$  integrin expression can be quantitated and on a second cell sample with the same type of cells,  $\alpha 1$  integrin expression can be quantitated. Alternatively, the level of  $\alpha 1$  and/or  $\alpha 2$  integrin expression can be determined using the same cell sample if the agent used to detect  $\alpha 1$  expression is detectably labeled with a first detectable label and

the agent used to detect  $\alpha 2$  expression is detectably labeled with a second detectable label. The first detectably labeled agent and the second detectably labeled agent are agents selected that can be detected and/or quantitated in the presence of one another.

In a preferred version, kidney tissue sections taken from diabetic patients are  
5 fixed in formalin and then treated with HCl and proteinase K. A first probe specific for  
 $\alpha 1$  integrin is a 3.9 kb fragment from 5' end through EcoR1 site near base 3900 probe  
including a sequence as shown in Figure 2 of Ignatius et al. (*supra*). This probe is  
labeled with  $^{32}P$  or  $^{35}S$  or other suitable labels known in the art including, but not  
limited to, fluorescent labels, biotinylated labels, or other radio labels and the like. The  
10 probe is incubated with the section as described previously. A second section taken  
from the same tissue sample is treated in the same manner but incubated with a probe  
specific for  $\alpha 2$  integrin expression. In a preferred embodiment, a probe specific for  $\alpha 2$   
integrin expression is a 1.8 kb fragment from 5' end through EcoR1 site near base 1800  
that includes a sequence as shown in Figure 2 of Takada et al. (*supra*). Both probes are  
15 labeled with  $^{32}P$  or  $^{35}S$ . The probe is incubated with the section overnight at 50°C and  
then for 4 days at room temperature. The sections are then developed for  
autoradiography. The number of grains per cell are counted for about 10-20 glomeruli  
and tubules. The total counts for  $\alpha 2$  integrin expression vs.  $\alpha 1$  integrin expression are  
compared. An increase of about 40% in  $\alpha 2$  integrin and a 30-40% decrease of  $\alpha 1$   
20 integrin may indicate a patient is at greater risk for developing diabetic nephropathy.

In an alternative version, the level of expression of  $\alpha 2$  integrin is compared with  
the  $\alpha 1$  expression which can be determined using *in situ* PCR or competitive reverse  
transcriptase PCR. Primers specific for  $\alpha 1$  and  $\alpha 2$  integrin expression can be prepared  
as described previously. For competitive reverse transcriptase PCR, RNA extracted  
25 from different cell types obtained from diabetic patients will be reverse transcribed to  
generate cDNA. The cDNA will be mixed with the various concentrations of  
competitive template amplified by the PCR method. After degradation of competitive  
cDNA with restriction enzyme, amplified cDNA will be subjected to electrophoresis in  
2% agarose gel, electrotransferred to a nylon membrane, UV cross-linked to the  
30 membrane and hybridized with a  $^{32}P$ -labeled probe. Autoradiographs will be used to  
quantify the label bound to the cDNA using amount of label bound to samples  
containing target cDNA alone as compared to samples also containing competitor

cDNA to arrive at the target cDNA concentration. For *in situ* PCR, a method has been described previously. The change in  $\alpha 1$  and  $\alpha 2$  integrin expression can be quantitated by counting the number of grains per cell in control vs. diabetic cells.

5        Optionally, for each of the detection methods for  $\alpha$  integrin subunits, the level of integrin subunit expression can be compared to expression of a control. The control is selected to be a protein expressed at the same levels in both normal and diabetic cells.  
*The control is not always the same*  
The control protein is also selected to be one that is expressed at sufficient levels for easy detection and quantitation. The level of expression of  $\alpha 1$  and  $\alpha 2$  integrin expression can each be compared to that of the level of the control RNA expression in the cells. The level of RNA expression of  $\alpha 1$  integrin or  $\alpha 2$  integrin can be divided by the level of expression of the control RNA to normalize the values to the level of control expression in a particular cell sample. The level of expression of the control protein is detected and quantitated using the same method as  $\alpha 1$  or  $\alpha 2$  integrin expression. The preferred control protein is a cell surface HLA determinant.

10      15     Optionally, the levels of  $\alpha 3$ ,  $\alpha 5$ , or beta-1 integrin subunit expression can be analyzed as described above. The level of  $\alpha 3$ ,  $\alpha 5$ , or beta-1 integrin expression in cells such as kidney tissue can be detected and quantitated as described for  $\alpha 1$  and  $\alpha 2$  integrin expression including *in situ* hybridization, *in situ* PCR, immunofluorescence and the like. Other cell types can be analyzed as described above, including fibroblasts and blood cells. Antibodies specific for  $\alpha 3$ ,  $\alpha 5$ , and beta-1 can be prepared as described by Wayner et al. cited *supra*.

20      25     A DNA sequence encoding  $\alpha 3$  integrin has been described in Takada et al., *J. Cell Biol.* 115:257 (1991). A probe specific for cDNA sequence encoding  $\alpha 3$  integrin subunit is a 1.4Kb Sal I fragment containing 5' untranslated and amino terminal coding sequences for  $\alpha 3$  subunit of integrin. DNA sequences encoding  $\alpha 3$ ,  $\alpha 5$ , and beta-1 integrin can be utilized to form primers and probes as described previously.

30      The level of  $\alpha 3$ ,  $\alpha 5$ , or beta-1 integrin expression is increased about 15 to 100% compared with cells from age matched nondiabetic controls. It is believed that an increase in  $\alpha 3$ ,  $\alpha 5$ , or beta-1 integrin subunit expression may also identify patients that have an increased risk of developing diabetic nephropathy or that have early signs of diabetic nephropathy.

This invention also relates to methods for detecting alterations in integrin subunit expression, particularly  $\alpha 1$  and/or  $\alpha 2$  integrin subunit expression by obtaining a cell sample from a patient, processing the sample to detect alterations in integrin subunit expression as compared to integrin expression in samples from age matched normal controls, detecting levels of integrin expression and determining if these levels are altered relative to controls.

This method is useful for predicting individuals at risk for developing pathologies associated with altered cell matrix deposition, including but not limited to renal nephropathy. In preferred embodiments of this invention, the tissues used to detect altered  $\alpha 1$  and/or  $\alpha 2$  integrin expression include kidney biopsies, skin biopsies and blood cells including polymorphonuclear cells, monocytes, and other cells expression integrin subunits. Biopsied tissue can be further separated into its cellular components or processed as tissue sections for *in situ* hybridization techniques, and/or for immunodiagnostic techniques including immunofluorescence and immunoperoxidase staining.

The cellular components of the biopsied tissue can be cultured for *in vitro* studies including Northern procedures, PCR techniques, immunofluorescent techniques and/or *in situ* hybridization techniques. Alternatively, cells can be separated and analyzed by flow cytometry, immunofluorescence, processed for PCR or for any of a variety of techniques discussed throughout this disclosure.

While blood cell components are preferably separated from the whole blood sample using methods well known in the art. Individual cells are separated, where necessary, using techniques such as those of Ng, et al. (*supra*), and Baron, et al. *Clin. Sci.* 37:205-219, 1990. Preferably the samples are tested using *in situ* hybridization methods. Where the amount of tissue available is fairly small, PCR-enhanced *in situ* hybridization can be used.

The present invention is also directed to a kit to detect alterations in integrin subunit expression, particularly  $\alpha 1$  integrin and/or  $\alpha 2$  integrin subunit expression in a patient sample. A variety of kits are contemplated to encompass a variety of methods. These kits optionally include reagents to process a tissue or cell sample for the technique employed by that particular kit. By example, a kit for PCR or PCR enhanced *in situ* hybridization can include reagents to process the cell sample or section and

isolate the RNA (for PCR). It will also contain suitable primers to amplify the target sequence and additional probes, if necessary, to detect the desired nucleic acid fragments as well as buffers and reagents for the polymerase chain reaction and the buffers and emulsions required to develop the silver granules, and the like, for *in situ* hybridization methods. Other kits can alternatively include reagents for immunofluorescence using antibodies to the integrin subunits and/or probes, primers and reagents for modifications of *in situ* or PCR *in situ* hybridization methods.

All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference.

It will be apparent to one of ordinary skill in the art that many changes and modifications can be made in the invention without departing from the spirit or scope of the appended claims.

**Example 1**  
**Effect of High Glucose on the Synthesis and**  
**Cell Surface Expression of Integrin Receptors**  
**by Cultured Mesangial Cells**

20

Cell lines and culture conditions

Human mesangial cells (HMC) were isolated from 19-22 week old fetal kidney tissue or adult tissue as previously described (Striker and Striker, *J. Lab. Invest.* 53(2):122-131, 1985). Cells were cultured at 37°C in an environment of 95% air and 5% CO<sub>2</sub> and in media composed of MEM (Sigma, St. Louis, MO) containing 5 or 25 mM glucose, 20% FBS, 15mM Hepes, penicillin (100 U/ml), streptomycin (100mg/ml), and amphotericin (25mg/ml). Cells were cultured in the two different conditions for at least two passages before they were used for experiments. Cells were released from their tissue culture flasks for passaging or for use in experiments, by washing twice with 1 mM EDTA in HBSS and then treating with 0.05% trypsin and 1 mM EDTA in HBSS for 1 min. Cells between passage 4 and 9 were used in experiments.

The cells were grown in T-75 flasks until 75-80% confluent. For the adhesion and immunoprecipitation analyses, cells were metabolically labeled for 18 hours with 0.5 mCi of [<sup>35</sup>S]-methionine per T-75 flask. [<sup>35</sup>S]-methionine was obtained from Du Pont/NEN, Boston, MA.

5

Monoclonal antibodies (Mabs) to integrin receptors

Mabs to the integrin receptors  $\alpha 3$  (P3D11),  $\alpha 5$  (P3D10) and  $\beta 1$  (P5D2) can be produced as previously described (Wayner et al., *J. Cell. Biol.* 121(5):1141 (1993)) and are available from EA Wayner, Fred Hutchinson Cancer Center, Seattle, WA. The 10 antibodies were characterized by sequential immunoprecipitation with known Mabs directed against these integrin receptors (P1B5, P1D6, P4C10) available from EA Wayner. Other Mabs  $\alpha 2$  (P1H5),  $\alpha 4$  (P4G9) and  $\beta 2$  (P4H9) were previously described (Wayner et al., cited *supra* 1993) and are available from EA Wayner, Fred Hutchinson 15 Cancer Center, Seattle, WA. TS2/7 was provided by Dr. Martin Hemler (Dana Farber Cancer Institute, Boston, MA).

SR84 supernatant was used as a function-blocking anti- $\alpha 1$  Mab in inhibition experiments. SR84 is available from Dr. D.O. Clegg (Univ. of California, Santa Barbara, CA). ( $\alpha 6$ ) G0H3 was purchased from AMAC Inc., Westbrook, ME. In addition monoclonal antibodies to  $\alpha 1$  and  $\alpha 2$  integrin were obtained from Telios 20 Pharmaceuticals (San Diego, CA). Hybridoma culture supernatant or ascites fluid were used for immunoprecipitation, flow cytometry and inhibition experiments. A Mab directed to a cell surface HLA determinant was used as a negative control (W6/32, HB95: American Type Culture Collection, Rockville, Maryland, USA). W6/32 bound to the surface of cultured mesangial cells but did not influence adhesion of cIV. SP2 25 myeloma culture supernatant was also used as a control.

Immunoprecipitation analysis of integrins from mesangial cell membranes

Mesangial cells metabolically labeled with [<sup>35</sup>S]-methionine were detached from flasks by treatment with trypsin (Sigma) for 2 minutes, washed three times with 30 phosphate-buffered saline (pH 7.4) and resuspended in PBS containing protease inhibitors (1 mM PMSF and 1 mM NEM). The radiolabeled cell membrane proteins were solubilized by adding lysis buffer (1% Triton X-100, 1 mM Calcium, 1 mM

PMSF, 1 mM NEM and PBS at pH 7.4) and incubating for 60 minutes at 4°C. Insoluble material was separated by centrifugation at 10,000 rpm for 30 minutes.

The supernatant was transferred and 10 µl was tested for radioactivity ( $\geq 10^7$  cpm/per antibody being assayed was considered to be adequate for 5 immunoprecipitation). The lysate was precleared once with fetuin-agarose which was removed by centrifugation at 10,000 rpm for 15 minutes. This was followed by three preclears with protein A agarose bound to rabbit anti-mouse IgG, the last preclear was done overnight.

For immunoprecipitation, the cell lysate (equal counts of lysate for cells in 5 and 10 25 mM glucose were used) was incubated with the monoclonal antibodies to be tested, pre-bound to rabbit anti-mouse protein A-agarose. Myeloma culture supernatant was used as a negative control. Anti-HLA antibody (W6/32) was used as a control for loading. After an overnight incubation at 4°C, the agarose beads were washed five times and bound material was eluted by boiling for 5 minutes in SDS.

15 The eluted material was analyzed by loading lysate from each permutation on a 7.5% non-reducing SDS-PAGE gel and labeled proteins were visualized by autoradiography. The fluorograms were scanned with a Macintosh Quadra 840 computer using the NIH Image 5.1 Program, and the optical density of the bands was red after subtracting the background. The O.D. was corrected using the lanes 20 immunoprecipitated with W6/32. Immunoprecipitation assays were performed three times for each growth condition of mesangial cells.

Immunoprecipitates were obtained with anti-integrin monoclonal antibodies from detergent extracts of metabolically labeled human kidney mesangial cells grown in 5 (low) or 25 mM (high) glucose. Equal counts of membrane proteins were 25 immunoprecipitated to compare the level of integrin receptors of mesangial cells under the two growth conditions of low or high glucose levels.

Cells grown in 25 mM glucose have a higher specific activity of labeling than cells in 5 mM glucose. To overcome this difference and permit a comparison of the band intensity on immunoprecipitation equal counts of cell lysate from the two 30 populations were immunoprecipitated with the antibody. Densitometry and statistical analysis of three experiments was performed, the data normalized to the HLA control and expressed as an O.D. ratio of cells grown in high glucose (HG) to cells grown in

low glucose (LG), for three experiments, with (LG = 1). Cells were labeled with [<sup>35</sup>S]-methionine, the cells were harvested, and solubilized. Samples were incubated with antibody and equal counts of cell lysate from the two cell populations were immunoprecipitated with equal amounts of antibody.

5       The control indicated that there were comparable amounts of cell surface HLA determinant precipitated from each sample. W6/32, a Mab to cell surface HLA determinant was used as a negative control. Other antibodies used included an anti- $\alpha$ 1 antibody (TS2/7) and an anti- $\alpha$ 2 antibody (P1H5). In total 5 mM and 25 mM glucose exposed cell extracts were immunoprecipitated side by side 3 times.

10      The  $\alpha$ 1 subunit band was clearly discernible at 180 kD in cell samples exposed to 5 mM of glucose and was associated with a  $\beta$ 1 band (116 kD). No  $\alpha$ 1 band could be seen in the 25 mM treated cell sample. In contrast, the  $\alpha$ 2 subunit band was more prominent in cell samples exposed to 25 mM glucose and appeared as a band at 130 kD.

15      The 130 kD  $\alpha$ 2 band was present in 5 mM glucose but was significantly less intense than the 25 mM glucose treated samples.

20      The cell lysates were also incubated with the following antibodies including: SP2 myeloma culture supernatant; anti- $\beta$ 1 (P5D2), anti- $\beta$ 2 (P4H9), anti- $\alpha$ 2 (P1H5), anti- $\alpha$ 3 (P3D11), anti- $\alpha$ 4 (P4G9), anti- $\alpha$ 5 (P3D10) and anti- $\alpha$ 6 (G0H3). Results were interpreted from three independent experiments. Immunoprecipitation of  $\alpha$ 3- $\alpha$ 6 and  $\beta$ 1 integrin subunits was performed on cells from the two growth conditions. Subunits  $\alpha$ 4 and  $\alpha$ 6 were not detected in either cell population. The antibody to the  $\beta$ 1 subunit precipitated a 116 kD protein, the  $\beta$ 1 subunit, and also a precursor  $\beta$ 1 band at 105 kD. The  $\alpha$ 3 and  $\alpha$ 5 subunits were seen at  $\approx$  130 kD with the associated  $\beta$  subunit at 116 kD, in both cell populations.

25

#### Flow cytometry

Cell surface expression of integrin subunits by cultured human mesangial cells was evaluated by indirect immunofluorescence staining and flow cytometry. Mesangial cells were released with trypsin, washed and resuspended in FACS buffer (HBSS, 2% goat serum, 0.02% sodium azide). An equal number of cells,  $2 \times 10^5$  were added to each vial.

The cells were incubated with primary antibody for one hour at 4°C and washed once with 1 ml FACS buffer. The secondary antibody was then added in a total volume of 0.5 ml FACS buffer and incubated for 30 minutes at 4°C. The cells were again washed in 1 ml of FACS buffer and resuspended in 0.5 ml of 2% formaldehyde.

5       The data was analyzed using CONSORT 30 software on a FACScan (Becton Dickinson, Mountain View, CA). Positive fluorescence was determined on a four decade log scale and fluorescence (log F1) was expressed as the mean channel number of 5,000 cells. Cell surface expression experiments were performed in duplicate with each antibody, at least three times with each growth condition of mesangial cells.

10      Densitometric scanning of the fluorograms generated from metabolically labeled cells indicated that the synthesis of the  $\beta 1$  (12%),  $\alpha 3$  (14%) and  $\alpha 5$  (19%) were moderately increased upon growth in 25 mM glucose. Growth in 25 mM glucose dramatically decreased synthesis of the  $\alpha 1$  subunit (39% reduction in intensity) while synthesis of  $\alpha 2$  was considerably increased (42%).

15      These changes in metabolic activity were paralleled by a similar change in the cell surface integrin phenotype of mesangial cells grown in high glucose. To assess the effect of different glucose concentrations in the medium on the levels of mesangial cell surface integrin receptor expression cells in each glucose treatment population were stained for immunofluorescence and processed for flow cytometry. Mean channel fluorescence (MCN) values of integrin subunit expression were obtained from 3 experiments. Within each experiment the ratio of MCN for cells grown in high glucose (HG) to cells grown in low glucose (LG), denominator = 1 was calculated.

20      Cell surface expression of the following integrin subunits was increased by growth in high glucose:  $\beta 1$  (24%),  $\alpha 2$  (26%),  $\alpha 3$  (18%), and  $\alpha 5$  (19%). The decrease in the synthesis of  $\alpha 1$  was reflected in a concomitant decrease in cell surface expression (33% reduction in specific staining). The  $\alpha 4$  and  $\alpha 6$  subunits were not detectable in cultured mesangial cells either by immunoprecipitation or flow cytometric analyses.

25      Mesangial cells grown in high glucose (for at least 2 passages) were returned to control media (5 mM glucose), again for at least 2 passages. A flow cytometric analysis of these cells revealed a reversion to "low glucose" type. The expression of  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$  and  $\beta 1$  were decreased while the expression of  $\alpha 1$  increased (data not shown).

**Example 2**  
**Adhesion of Cultured Mesangial Cells to**  
**Type IV Collagen (cIV); Effect of High Glucose**

5      Cell adhesion to collagen IV (cIV)

The cells were detached from culture flasks by incubation with trypsin 0.05% and EDTA 0.02% for two minutes at 37°C, then washed twice with DMEM and resuspended to the appropriate concentration in binding buffer (DMEM, 25 mM HEPES, 2 mg/ml BSA at pH 7.4). 48 or 96 well plates were coated overnight at 29°C with cIV in serial dilutions starting from 100 µg/ml (5 µg/96 well or 20 µg/48 well). Under these conditions approximately 50% of the cIV adhered. To block the remaining reactive sites the plates were treated with 200 µl of BSA at 2 mg/ml for 2 hours at 37°C. 50 µl of suspension containing 5000 cells (96 well plates) or 100,000 cells (48 well plates) was added per well. The plates were incubated at 37°C in a humidified incubator for approximately 30 minutes. The non-adherent cells were removed by washing three times with binding buffer and then 100 µl of "lysis" buffer (0.5 NaOH, 1% SDS in distilled water) was added to each well for 30 minutes at 60°C. The lysate was transferred to scintillation vials and counted. The data was expressed as a percentage of the total input cpm. Cell adhesion assays were performed in triplicate, at least three times for each growth condition.

Cells grown in medium containing 25 mM glucose adhered significantly better than cells in 5 mM glucose. Adhesion increased with coating concentration of cIV and was saturated at 25 µg/ml for both cell populations.

25      Inhibition of cell adhesion with monoclonal antibodies

Since growth in high glucose appeared to alter the synthesis and expression of the integrin receptors  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  which have been reported to be involved in cell adhesion to collagen, (Wayner and Carter, *J. Cell. Biol.* 105:1873 (1987)), we examined the effects of glucose on the ability of mesangial cells to adhere to cIV.

30      Monoclonal antibody inhibition of  $^{35}\text{S}$ -methionine labeled human mesangial cells grown in 5 mM glucose to cIV was assessed. Briefly, 96 or 48 well plates were coated with 50 or 200 µl of cIV at 2.5 µg/ml, overnight at 29°C. The plates were incubated with 2% BSA in PBS to coat remaining reactive sites on plastic for 2 hours,

and then hybridoma culture supernatant or ascites containing 10 µg/ml of antibody were added to each well, followed immediately by the cells. After 30 minutes non-adherent cells were washed off and adherent cells were quantitated. Results were obtained from 3 experiments. SP2 myeloma culture supernatant of W6/32 were used as negative controls. A quantitative ELISA was used to determine the concentration of antibody in the hybridoma culture supernatant or ascites.

In each case, the concentration of monoclonal antibody (Mab) was determined relative to a standard curve generated with an isotype-matched control mouse IgG. The concentration of antibody required to saturate the binding sites on human mesangial cells was determined by flow cytometry. The concentration of the antibodies used in the inhibition assays were well above the saturating concentration as determined by flow cytometry. Data were expressed as the percent of maximal binding observed in the presence of W6/32 antibody. Inhibition experiments were performed at least three times, in triplicate, for each growth condition with the various antibodies.

Mesangial cells grown in high glucose (25 mM) adhered better to cIV than cells grown in low glucose (5 mM). Results indicated that adhesion increased with coating concentration of collagen IV and saturated at about 25 µg/ml for both cell populations.

In order to examine the activity of collagen receptors expressed by mesangial cells grown in high glucose, we performed adhesion experiments in the presence of well characterized neutralizing antibodies directed to various  $\beta 1$  integrin subunits. A panel of antibodies was used all of which have been reported to inhibit the adhesion of cells to various substrates (Wayner and Carter, cited *supra*, 1987; Wayner et al., cited *supra*, 1993). Antibodies were used at saturating concentrations as determined by immunofluorescence staining and flow cytometry. In the competition experiments, the following criteria were selected to promote half-maximal binding of mesangial cells: 2.5 µg/ml cIV and a short term assay (less than 30 min). The ability of neutralizing Mabs to inhibit mesangial cell adhesion to cIV was examined in low (5 mM) or high glucose (25 mM) containing media.

To test Mab-mediated adhesion inhibition of mesangial cells grown in 5 mM glucose or 25 mM glucose to collagen IV,  $^{35}$ S-methionine labeled human mesangial cells were seeded in 48 well plates (100,000 cells/well) coated with 200 µl cIV (2.5 µg/ml, overnight at 29°C). Mab's anti- $\alpha 1$ , SR84, anti- $\alpha 2$ , P1H5, anti- $\beta 1$ , P5D2 and

SR84 and P1H5 together, were added to the wells before seeding with cells. Adhesion in the presence of W6/32 was used as a control. After 20 minutes non-adherent cells were washed out and adherent cells quantitated. The data was expressed as a percentage of the binding in the presence of W6/32. and the two cell populations were normalized  
5 by using the binding in the presence of HLA antibody to represent 100% and the inhibition by other antibodies was calculated as a percentage of binding in the presence of HLA.

The results indicated that the  $\alpha 1\beta 1$  integrin receptor had a reduced role (\* $p < 0.001$ ) for cells grown in 5 mM glucose as compared with 25 mM glucose. Of the  
10 antibodies examined, only Mabs directed to the  $\alpha 1$  (SR84),  $\alpha 2$  (P1H5) or  $\beta 1$  (P5D2) integrin subunits inhibited the binding of mesangial cells to cIV. When mesangial cells were grown in either low or high glucose, adhesion to cIV could be almost completely inhibited with Mabs to  $\beta 1$  (P5D2) or a combination of  $\alpha 1$  (SR84) and  $\alpha 2$  (P1H5).

The relative effects of the neutralizing Mabs directed against the  $\alpha 1$  and  $\alpha 2$  subunits varied depending on whether mesangial cells were grown in low or high glucose. In 5 mM glucose the Mab to the  $\alpha 1$  subunit of integrins resulted in more inhibition ( $\approx 50\%$ ) than in 25 mM glucose ( $\approx 20\%$ ) ( $p < 0.001$ ). This is consistent with the presence of significantly more  $\alpha 1$  integrin on the surface of cells grown in 5 mM glucose. Alternatively, in 5 mM glucose the Mab to the  $\alpha 2$  subunit resulted in less inhibition ( $\approx 60\%$ ) than in 25 mM glucose ( $\approx 75\%$ ) ( $p < 0.001$ ). Mab's against the  $\alpha 3$ ,  
20  $\alpha 4$ ,  $\alpha 5$  and  $\alpha 6$  subunits did not inhibit adhesion (data not shown).

These data demonstrate that under low glucose growth conditions, mesangial cells use  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  integrins to bind cIV coated surfaces. However, cells grown in high glucose, appear to rely more on the  $\alpha 2$  subunit complexed with  $\beta 1$ . The results of  
25 these functional studies are consistent with the observed alterations in the integrin cell surface phenotype discussed in Example 1.

**Example 3**  
**Localization of  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  Integrin Receptors**  
**Localization of  $\alpha 1$  integrin in focal adhesions**

30 Glass cover slips were coated with 50  $\mu$ l of cIV at 2.5  $\mu$ g/ml, overnight at 29°C. The coated areas were "blocked" for two hours with BSA at 2 mg/ml, in PBS. Human

- mesangial cells were processed as before, seeded on each spot of cIV in 50 µl of binding buffer (2500 cells) and allowed to adhere for 5 hours at 37°C. The unbound cells were washed off with PBS. Adherent cells were fixed with 2% paraformaldehyde in HBSS for 30 minutes followed by permeabilization with 0.5% Triton X-100 for 2 minutes.
- 5       The cells were blocked again with PBS following which 200 µl of hybridoma culture supernatant containing anti- $\alpha$ 1 antibody (TS1/7) was added to each spot and incubated at room temperature for 1 hour. The coverslips were then thoroughly washed and rhodamine-conjugated goat anti-mouse antibody (1:100) (Boehringer Mannheim, Indianapolis, IN) was added for one hour. The coverslips were again washed and
- 10      incubated with anti-vinculin antibodies (Sigma, St. Louis, MO) preconjugated (Quicktag, FITC labeling kit, Boehringer Mannheim, Indianapolis, IN) to FITC labeled goat anti-mouse antibody for 1 hour at room temperature. The coverslips were finally washed, mounted on glass slides and viewed for focal adhesions by co-localization of vinculin with  $\alpha$ 1 integrin.
- 15      Staining of normal human adult kidneys for the presence of  $\beta$ 1 integrins
- Normal human adult kidney tissue was snap frozen in liquid nitrogen and sections were prepared with a cryostat at 5 µm intervals. The sections were stained using an anti-mouse Vectastain Elite Kit (as described by Wayner et al., 1993) with diamino benzene (DAB) as the chromogen. The following mAbs were used:  $\alpha$ 1 (TS2/7),  $\alpha$ 2 (P1H5),  $\alpha$ 3 (P3D11),  $\alpha$ 4 (P4G9) and  $\beta$ 1 (P5D2). These monoclonal antibodies are available from the following sources and stained the following histological areas as was demonstrated in these studies:
- 20       $\alpha$ 1 (TS2/7)     Martin Hemler, Dana Farber Cancer Center, Boston, MA.  
                        Stained mesangium.
- 25       $\alpha$ 2 (P1H5)     EA Wayner, Fred Hutchinson Cancer Center, Seattle, WA  
                        Stained mesangium.
- 30       $\alpha$ 3 (P3D11)    EA Wayner, Fred Hutchinson Cancer Center, Seattle, WA  
                        Stained the mesangium,  
                        endothelium, visceral and Bowman's  
                        epithelium and capsule.
- 35       $\alpha$ 4 (P4G9)     EA Wayner, Fred Hutchinson Cancer Center, Seattle, WA  
                        Did not stain glomeruli.

$\beta 1$  (PSD2) EA Wayner, Fred Hutchinson Cancer Center, Seattle, WA  
Stained mesangium, endothelium,  
visceral epithelium, Bowman's  
epithelium and capsule.

5

Normal mouse IgG (all isotypes) was used as a negative control.

These studies demonstrated the presence of  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  integrin receptors in focal adhesions. Focal adhesions are observed when cells spread in culture on matrix components such as collagen IV, fibronectin or laminin. Integrins cluster at the site of 10 focal adhesions on the cell surface with intracellular fibers such as vinculin staining at these locations within the cell periphery. (see Hynes, et al. *Cell* 69:11-25, 1992 and Burridge, et al. *Ann. Rev. Cell Biol.* 4:487-525, 1988). This supports the hypothesis that 15 mesangial cells use  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  integrin receptors to bind to cIV. It has been well established that when a particular integrin receptor is engaged by a specific ligand it can be detected in focal contacts co-localized with certain components of the cytoskeleton 20 such as vinculin. Therefore, we asked whether mesangial cells could localize  $\alpha 1$  (or  $\alpha 2$  and  $\beta 1$ ) to focal adhesions when seeded on cIV coated substrates.

$\alpha 2$  or  $\beta 1$  could be detected in focal contacts on cIV regardless of whether 25 mesangial cells were grown in either low or high glucose. Additionally, when mesangial cells were grown in 5 mM glucose and subsequently seeded on cIV coated surfaces,  $\alpha 1$  could also be co-localized with vinculin within several focal contacts by dual-label immunofluorescence staining. It is believed that cIV binding in cells maintained in low glucose engages both the  $\alpha 1$  and  $\alpha 2$  subunits.  $\alpha 1$  could be detected in only some of the focal adhesions stained by vinculin. As a control,  $\alpha 1$  was not 30 detected in focal contacts when mesangial cells were seeded onto fibronectin coated surfaces regardless of the glucose concentration of the cell culture media.

Immunohistochemical staining of integrin receptor subunits in normal human adult and fetal kidney revealed that both  $\alpha 1$  and  $\alpha 2$  could be localized within the mesangium. The  $\alpha 1$  receptor was diffusely expressed throughout the mesangium whereas the distribution of  $\alpha 2$  was more limited and focal. Also consistent with the results we obtained with cultured mesangial cells,  $\beta 1$  and  $\alpha 3$  were intensely expressed throughout the mesangium, while  $\alpha 4$  could not be detected in either fetal or adult mesangium.

**Example 4****Alterations in RNA Production in Human Mesangial Cells  
cultured in High and Low Glucose Concentrations**

5 Our efforts have concentrated on finding a way to predict, at early stages after the onset of diabetes, the subjects who will later develop nephropathy. We focused on a major hallmark of diabetic nephropathy, that of mesangial expansion. We first examined mesangial cells in culture, since these cells secrete their surrounding matrix, which is expanded in diabetes; however, biopsied tissue can be treated in the same 10 manner, as will be understood by those skilled in the art. The matrix consists primarily of collagen IV.

Primary cultures of human mesangial cells undergo several phenotypic changes in response to elevated glucose concentrations and glucose-modified ("glycated") collagen IV. These changes included altered cell interactions with the collagen matrix. 15 In elevated glucose concentrations, the  $\alpha 1$  subunit underwent a substantial decrease, concomitant with an increase of the  $\alpha 2$  integrin subunit. This change was observed with immunoprecipitation and flow cytometry. Further studies with Northern analysis and *in situ* hybridization of the cultured mesangial cells confirmed the integrin reversal. In the studies employing Northern analyses, separate samples of total RNA were isolated from 20 the mesangial cells on each culture plate or alternatively from rat kidneys (see Example 5, below) by a single-step method using RNA STAT-60<sup>TM</sup> isolation reagent (TEL-TEST "B", INC., Friendswood, TX) according to the manufacturers directions. Briefly, the cells were lysed with RNA STAT-60<sup>TM</sup> solution by repetitive pipetting; the tissues were cut into small pieces and homogenized in the RNA STAT-60 solution with a high-speed 25 tissue homogenizer (Polytron CH6005, Luzern, Switzerland). The nucleic acid mixture was extracted with 0.2 ml chloroform per 1ml of the RNA STAT-60<sup>TM</sup> solution. Total RNA was precipitated for 10 min at -80°C in isopropanol, and the pelleted RNA was redissolved in TE buffer. The total RNA was free of DNA and proteins and had a 260/280 wavelength ratio > 1.8.

30 *Northern blot analysis*-The RNA samples were denatured in formaldehyde gel-running buffer (20 mM MOPS, 8 mM sodium acetate, mM EDTA, at pH 7.0) containing 6% formaldehyde and 50% formamide by heating at 65°C for 15 min. For each sample 20 mg of RNA was mixed with 6x loading buffer (50% glycerol, 1 mM

EDTA, 0.25% bromphenol blue, 0.25% Xylene cyanol FF), loaded on a 1% agarose gel submerged in 6% formaldehyde running buffer, and run at 3-5 V/cm for 3-4 hours. RNA was transferred from the agarose gel to a nylon membrane (Boehringer Mannheim, Indianapolis, IN) by capillary elution and immobilized by UV cross-linking  
5 (Stratalinker UV; Stratagene, La Jolla, CA). The membranes were then incubated in prehybridization solution containing 50% formamide, 5xSSC, 0.02% SDS, 0.1% N-lauroylsarcosine, 2% blocking reagent (Boehringer Mannheim), and 20 mM sodium maleate (pH 7.5) for >3 hours at 42°C. Radiolabeled probes (see Example 5) for the integrin subunits or controls were then added to the prehybridization solution and  
10 hybridization was performed overnight at 42°C (for cDNA probe) or 50°C (for antisense RNA probe). After hybridization, the membranes were initially washed in 2x SSC, 0.05% SDS for 10 minutes at room temperature and then washed for an additional 40 minutes at 42°C (for cDNA probe) or 60°C (for antisense RNA probe). Membranes were then exposed to X-ray film (X-Omat RP; Eastman Kodak Co., Rochester, NY) for  
15 1 day at -80°C. After being stripped of previous probes by heating in 0.2x SSC, 0.5% SDS for 10 min at 100°C, the membranes were reprobed as described above. Images of autoradiograms were captured and digitized using a CCD video camera module interfaced with a microcomputer (Macintosh IIcx: Apple Computers Inc., Cupertino, CA) and analyzed using image processing software (NIH Image 1.55b77: public  
20 domain).

Cells grown in 25 mM glucose expressed lower levels of  $\alpha 1$  integrin than seen in an equivalent amount of RNA from cells grown in 5 mM glucose. Densitometric analysis demonstrated an  $\approx$ 30% decrease upon averaging the values from four samples. Similar analysis demonstrated  $\approx$ 30% increase in  $\alpha 2$  integrin expression in cells grown in  
25 25 mM glucose.

#### Example 5

#### *In Situ* Hybridization Detecting Expression of Integrins in Kidney Sections Taken at Various Times

##### After Onset of Diabetes

30 The expression of  $\alpha 1$  and  $\alpha 2$  integrin receptors was examined in rat kidney sections after the onset of diabetes.

The *in situ* hybridization approach was used to examine kidney sections of streptozotocin-diabetic rats, 2.5 months after induction of diabetes. At this time interval, glomerular changes were still minimal. The streptozotocin-induced diabetic rat model mimics human changes of mesangial expansion and glomerular basement membrane thickening in late nephropathy and is an accepted model for diabetes and nephropathy.

Female non-pregnant Sprague-Dawley rats were obtained from Brithwood, Minneapolis, MN. The animals weighed 190-210 g at the beginning of the experiments and were given a 52mg/kg intraperitoneal dose of streptozotocin (STZ, Zanazar brand, 10 Upjohn Corp., Kalamazoo, MI) in calcium citrate and calcium carbonate Buffer (pH 4.5) to induce diabetes, while the controls were injected with the same amount of Hanks' balanced salt solution (pH 7.2). The animals were fed on standard rat chow (Purina laboratory chow # 5001. RFG PET@Supply Company, Plymouth, MN), and tap water *ad libitum*. Presence of diabetes was confirmed by detection of >400mg/dl nonfasting 15 plasma glucose levels 10 days post injection by tail vein bleeding using the glucose peroxide method (Beckman glucose analyzer, Beckman Instruments, Inc., Fullerton, CA).

Body weight was determined weekly, blood glucose levels were determined at 4 weeks after induction of diabetes, and on the day before the termination of the 20 experiment, which was 2.5 month from induction of diabetes. Urinary albumin excretion (UAE) was determined by radial immunodiffusion Mancini method, using goat IgG fraction against rat albumin (Cappel Cat. No. 55727) and purified rat albumin (Cappel Cat. No. 55952, Cappel Research Products, Durham, NC), according to previously published procedures (Mauer et al, *Diabetes* 27:959-64, 1978). Rats were 25 sacrificed at 2.5 months after diabetes induction and kidney tissue was perfusionally fixed by injecting freshly prepared 4% paraformaldehyde through the renal artery. This was followed by overnight fixation in 4% paraformaldehyde after removal from the body. The tissue was sectioned at 5 µm and placed on the silane-coated slides (Digene Diagnostics, Inc., Beltsville, MD) for *in situ* hybridization with probes for the α1 and α2 integrin subunits.

2.5 months after injection of STZ, diabetic rats weighted significantly less than controls, whereas their right kidney weight and serum glucose concentration were

significantly increased, as compared to the controls (see Table 1). Diabetic and non-diabetic rats demonstrated no significant difference in glomerular size and albumin excretion at 2.5 month after induction of diabetes (Table 1).

TABLE 1

TISSUE	CONTROL	DIABETIC	S/NS
Body Wt.(g)	390+/-10	200+/-20	S
Right Kidney wt. (g)	1.35+/-0.1	1.8+/-0.1	S
Plasma glucose (mg/dl)	140+/-25	760+/-150	S
Glomerular area	1.42+/-0.5	1.45+/-0.6	NS

5

A 5.4 kb human  $\alpha 2$  integrin cDNA clone (Takada, et al., 1989, *supra*) and a rat  $\alpha 1$  integrin cDNA clone (Ignatius et al, *supra*) in bluescript vector (Stratagene, La Jolla, CA) were used in these experiments. A 1.79 kb  $\alpha 2$  integrin cDNA fragment was restriction digested from the EcoRI site. Similarly, a 3.98 kb  $\alpha 1$  integrin cDNA fragment was obtained by restriction digestion from the EcoRI site.

10 cDNA fragments were purified by GENE CLEAN II kit (BIO 101, San Diego, CA) and labeled using the random primer labeling kit (Boehringer Mannheim, Indianapolis, IN) with  $P^{32}$ -dCTP (NEN) for Northern blotting and with  $S^{35}$ -dCTP (NEN) for *in situ* hybridization. GAPDH and sheep visna virus cDNA (PLV-KS) (Staskus et al, *Virology* 181:228-240, 1991) probes were used as the positive and negative controls respectively. The probes preferably had a specific activity of  $2 \times 10^8 - 1 \times 10^9$  dpm/ $\mu$ g.

15 By Northern blotting, compared to the controls, the diabetic kidneys expressed 113.5% more  $\alpha 1$ (IV) RNA, 46.5% more  $\alpha 3$ (IV) RNA, 54.8% less metalloproteinase-2 RNA (MMP-2, an enzyme that cleaves type IV collagen) and 246% more TIMP-1 RNA (a tissue inhibitor of metalloproteinases) with a  $p < 0.01$  in all cases as determined by ANOVA.

20 The expression of  $\alpha 1$  and  $\alpha 2$  integrin RNA was localized using a modification of a previously described method for *in situ* hybridization (Staskus et al. *supra*). 5 $\mu$ m tissue sections on silane-coated slides were fixed in the freshly prepared 4% paraformaldehyde for 10 min. The slides were pretreated with 0.2N HCl for 20 min, 0. 25 15 M Triethanolamine (TEA, Sigma, St. Louis, MO) for 15 min, 0.005% digitonin for 5 min, 3 mg/ml proteinase K (Sigma) for 15 min at 37°C, and 0.3% acetic anhydride -

O.1M TEA for 10 min. Hybridizations were performed under stringent hybridization conditions. Stringent hybridization conditions are defined in this specification as 50°C overnight, in 50% formamide, 0.6 M NaCl, 1x Denhardt's solution, 0. 17 mg/ml human COT<sup>RT</sup> DNA (GIBCO/BRL), 1 mg/ml poly A (Boehringer Mannheim), 10% (w/v) Dextran sulfate (Sigma), 0. 1 M dithiothreitol (DTT, Boehringer Mannheim), 1 mM EDTA, 0. 1 mM aurinitricarboxylic acid (ATA, Sigma) and S<sup>35</sup>-dCTP labeled cDNA probe. The next day, the slides were washed in 2x SSC-0.05% SDS for 60 min at 55°C (recipes for SSC and the like can be found in Sambrook, et al., *supra*); further washed in a high stringency washing buffer containing 50% formamide, 0.6 M NaCl, 1 mM EDTA, 5 mM DTT and 10 mM Hepes for 4 days at room temperature. After a brief rinse in 2x SSC, the slides were dehydrated in graded ethanol with 0.3 M ammonium acetate then dipped in Kodak NTB-2 emulsion and exposed for 5 days at 4°C.

After development the slides were stained with hematoxylin-eosin (Surgipath Canada, Inc., Winnipeg, Canada) and mounted. A ratio of the number of silver grains per cell was used to quantitate the results of *in situ* hybridization. Twenty glomeruli each were counted from each control and diabetic animal. Each glomerulus was assessed for: 1) glomerular area; 2) glomerular perimeter; 3) grains per glomerulus; and 4) number of cell nuclei per glomerulus.

The results were estimated as grains per cell nucleus and grains per glomerular area, as mean +/- SD of 5 animals (20 glomeruli each). (Haase, A.T., [1990]: *In situ hybridization*, CRC Press, 199-217; Nuovo, G.J., [1992] *PCR in situ hybridization, protocols and applications*, Raven Press). Groups were compared with the 2-tailed student t-test. Differences between groups were considered significant at p<0.05.

The results are illustrated in Fig. 1. Early after induction of experimental diabetes, the expression of the  $\alpha 1$  integrin subunit by glomerular cells was decreased compared to the control, whereas the expression of  $\alpha 2$  integrin was increased. The average counts, in diabetic glomeruli hybridized with the  $\alpha 1$  integrin probe, were significantly lower than control (Fig. 1). Also, the average counts, in diabetic glomeruli hybridized with the  $\alpha 2$  integrin probe, were significantly higher than control (Fig. 1).

Control animals at 2.5 month diabetes expressed on an average a significantly higher level of  $\alpha 1$  subunit integrin and significantly lower levels of  $\alpha 2$  subunit integrin using unbiased methods of selection of areas for study. The entire section was surveyed

for RNA grains, the regions of the Bowman's space and the background count were excluded by studying a commensurate area of the negative control stained tissue.

Compared to the control, glomerular cells (GC:endothelial, epithelial and mesangial combine) and/or tubular (proximal and distal epithelial) cells (TC) had 36%  
5 (GC) less grains for  $\alpha 1$  integrin; 86.4% (GC) more grains for  $\alpha 2$  integrin; 82(TC)-167%  
(GC) more grains for  $\alpha 1$ (IV); 107 (TC)-137% (GC) more grains for  $\alpha 3$ (IV); 63.6(GC)-  
65.3%(TC) less MMP-2.

10 The results of the present study clearly demonstrate that mesangial cells, when cultured in high glucose (25 mM) instead of normal/low glucose (5 mM) alter their RNA production for the integrin subunits  $\alpha 1$  and  $\alpha 2$ . Thus, this phenomenon is observed both at the level of protein and RNA production.

15 Furthermore, the results of our *in situ* hybridization and immunohistochemical staining experiments show that these changes can be detected in the mesangium of diabetic rat kidney and that human  $\alpha 2$  integrin subunit probes and rat  $\alpha 1$  integrin subunit probes are functional in both rat and human cells. Work by Mendrick and co-workers (*Lab. Invest.* 72(3):367-375, 1995) has shown that in the rat both integrins  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  of mesangial cells interact with collagen; as happens in the human mesangial cells. In the present study, the distribution of  $\alpha 1$  and  $\alpha 2$  integrin receptor subunit RNA was precisely localized by *in situ* hybridization to the different cell types of the  
20 glomerulus and surrounding tubules. Normal rat tissues expressed levels of the  $\alpha 1$  subunit and also the  $\alpha 2$  subunit RNA, as determined by counting the number ratio of silver grains/cell. However, the streptozotocin-induced diabetic animals had significantly lower levels of RNA for the  $\alpha 1$  subunit and significantly higher levels of  $\alpha 2$  subunit. A similar distribution of  $\alpha 1$  and  $\alpha 2$  subunit RNA (silver grains) was seen in  
25 the proximal and distal tubular epithelial cells. These data indicate that the distribution of cell surface integrin expression may be regulated by gene expression at the transcriptional level.

30 In summary, using *in situ* hybridization, similar results were seen in both mesangial cells *in vitro* and in glomeruli from tissue sections probed for the  $\alpha 1$  and  $\alpha 2$  integrin.

Early after induction of streptozotocin-diabetes in rats, substantial matrix-related gene expression changes occurred. For example,  $\alpha 1$  and  $\alpha 2$  integrin levels

changes, components of  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  integrin cell receptors for tIV (an important component of the renal extracellular matrix) underwent a reversal in levels with less  $\alpha 1$  and more  $\alpha 2$  integrin being present in glomeruli from kidneys of diabetic rats, when compared to the control. Expression of tIV was increased whereas the expression of  
5 MMP-2 which degrades tIV was substantially decreased. TIMP-1, an inhibitor of MMP-2 was increased. The observed matrix changes indicate an imbalance of tIV synthesis and turnover. This dysmetabolism of tIV, apparent in both the glomerular and tubular areas of the kidney, occurred before significant renal functional changes, or matrix accumulation out of proportion to renal enlargement, could be detectable. These  
10 changes could have a regulatory role in significant basement membrane thickening and mesangial expansion of diabetic nephropathy.

Collectively, the obtained data indicate that increased glucose concentration induces quantitative changes in receptor synthesis and cell surface integrin expression of human mesangial cells. In the diabetic, all cell systems are exposed to hyperglycemia  
15 and it is known that many cell and organ systems are affected by the disease; therefore, other cell types could similarly be used to assess changes in the levels of  $\alpha 1$  and/or  $\alpha 2$  integrin subunit expression as a measure of a predisposition to a variety of diabetic-induced pathologies. Kyu-Jin, et al. (*supra*) have noted alterations in integrin subunit expression in skin fibroblasts of diabetic patients. This information, in conjunction with  
20 the data discussed herein, indicates that altered levels of integrin subunit expression can be detected from a variety of integrin-expressing cells in diabetic nephropathy patients.

These results support the *in vitro* primary human mesangial cell culture data demonstrating that changes in cell surface integrin expression indicate the onset of nephropathic changes.  
25

#### Example 6

##### Detection of Altered Levels of $\alpha 1$ and $\alpha 2$ Integrin Subunit Expression in Humans using Blood and Tissue Samples

Patients with insulin-dependent diabetes mellitus (IDDM), individuals at risk for  
30 developing IDDM, patients with clinical diabetes nephropathy and healthy age matched volunteers are selected for studies to confirm the presence of altered  $\alpha 1$  and  $\alpha 2$  integrin subunit expression in integrin-producing cells. Clinical diabetic nephropathy is defined by the presence of persistent proteinuria (urinary AER > 300  $\mu$ g/day) in sterile urine of

patients with >10 yr duration of disease and concomitant retinopathy and is confirmed by the presence of classic glomerulosclerotic lesions on renal biopsy. Normal, nondiabetic individuals without a family history of hypertension serve as control subjects.

5 Patients were biopsied as follows: For skin biopsies, a biopsy is taken from the anterior surface of the left forearm by excision under local anaesthetic such as ethyl chloride, see Trevisan, et al. *Diabetes* 41:1239-45, 1992. The biopsy is optionally divided in half. With half of the tissue frozen immediately in liquid nitrogen and the other half placed in Hanks balanced salt solution. The frozen tissue is embedded in  
10 paraffin and processed for *in situ* hybridization as has been described above. A portion of the intact tissue is preferably immediately minced and processed for RNA isolation using techniques described above. Remaining minced tissue is gently digested with trypsin to obtain a cell suspension, washed in media containing serum to remove trypsin and plated onto tissue culture dishes containing 10% FCS supplemented DMEM with  
15 antibiotics.

Renal biopsies were obtained as follows. Patients should have normal blood pressures, normal coagulation values and platelet counts. Ultrasound was used to precisely localize the kidney. Ultrasound was also used to determine renal size, structural defects and post-void residual urine. Renal biopsies were performed on  
20 sedated patients using the Franklin modified Vim-Silverman or Truecut needles available from surgical supply suppliers. The biopsy specimens were immediately examined under a dissecting microscope to ensure that adequate samples of glomeruli were present for subsequent studies to quantitate integrin levels. Biopsied tissue was sectioned and processed for *in situ* hybridization as described in Example 5. In one  
25 example, renal samples from diabetic patients who did not show signs of microalbuminuria, but who had diabetic siblings with renal nephropathy were processed for *in situ* hybridization and PCR *in situ* hybridization. Renal samples from diabetic patients without a family history of nephropathy were also studied by PCR *in situ* hybridization to detect altered levels of integrin subunit expression.

30 PCR *in situ* hybridization is performed as follows. Sections are fixed as described in Example 5 and rinsed in RNase free water. The protocol used is that described by Nuovo, et al. (*Am. J. Surg. Pathol.* 17:683-690, 1993.) Cells are treated

with pepsin and DNase as described. cDNA synthesis is initiated by adding 10 $\mu$ l of a solution containing one or more of the following probes listed in a 5'-3' orientation with their SEQ ID NOS and their nucleic acid location on the respective integrin gene with reverse transcriptase (Perkin-ELmer, Norwalk, Conn.):

5

	<u><math>\alpha 1</math> integrin primer</u>	SEQ ID NO	NA location
	CCAGAGTCACTCTCACAGAG	5	2729-2748
	CACAGCGTACACGTACACC	6	1991-2009
	CACTTATAGACATCTCCAG	7	646-664
10			
	<u><math>\alpha 2</math> integrin primer</u>	SEQ ID NO	NA location
	CATCCATGTTGATGTCTG	8	1733-1750
	CATGTGATTCAACCGTCAG	9	894-910
15	GCATATTGAATTGCTCCGAATGTG	10	801-826

The resulting cDNAs are subjected to amplification containing a 1  $\mu$ M concentration (each) of one or more of the above primers with a paired primer located 5' to the primers provided above. Those skilled in the art will recognize that a variety of other primers could also be used from the  $\alpha 1$  and  $\alpha 2$  integrin gene sequence to similarly perform PCR *in situ* hybridization. The preferred primers paired with the above primers are provided below.

	<u><math>\alpha 1</math> integrin primer</u>	SEQ ID NO	NA location	SEQ ID Pair
25	GGCGTATGCACAACGCA	11	2261-2277	5
	GCGACAGCTGACCAGTCAGCA	12	1509-1529	6
	CACTCCTCCACAGCTCCT	13	251-268	7
	<u><math>\alpha 2</math> integrin primer</u>	SEQ ID NO	NA location	SEQ ID Pair
30	ACATGTACTCACTGG	14	1593-1608	8
	CTCACATGTGGTCCTCTG	15	433-451	9
	GTCCTGTTGACCTATCCACTGC	16	296-319	10

The SEQ ID Pair in the above table refers to the paired primer that provides amplification of the sequence positioned between the primer pairs on the respective integrin gene. The PCR products are detected by using an antidigoxigenin-alkaline phosphatase conjugate and the chromagen nitroblue tetrazolium (NBT)-5-bromo-4-chloro-3-indoylphosphate toluidinium (Salt) (BCIP). The counterstain nuclear fast red

is used to stain nuclei. Internal probes located within the nucleic acid regions amplified by PCR can also be used to identify the amplified fragments. Thus, based on the pairings provided above, oligonucleotide probes can be selected between regions 267-645, 1530-1990 and between 2278-2728 for the  $\alpha 1$  integrin gene and between regions 5 320-800, 452-893, 1607-1732 for the  $\alpha 2$  integrin gene and hybridized and stained following the *in situ* hybridization methods detailed in Example 5.

A blood sample is also taken from the patient and leukocytes are isolated from blood by centrifugation, followed by hypotonic shock of residual blood cells. The leukocytes are then processed for *in situ* hybridization as has been discussed in the 10 preceding examples.

#### Results:

PCR *in situ* hybridization with renal tissues demonstrated decreased  $\alpha 1$  and increased  $\alpha 2$  integrin subunits in the patient with diabetic neuropathy as compared with 15 control tissue.

Quantitative analysis of RNA grains per unit area of kidney glomeruli and tubules was performed by counting silver grains under epi-polarized light.

As shown in Table 2, both glomeruli and tubules of the diabetic neuropathy patient showed significantly decreased  $\alpha 1$  integrin levels as compared to the control, 20 whereas  $\alpha 2$  integrin levels were significantly increased as compared with control levels.

TABLE 2

Sample	Glomeruli <sup>a</sup>		Tubules <sup>a</sup>	
	$\alpha 1$	$\alpha 2$	$\alpha 1$	$\alpha 2$
Control	156	83	136	101
Diabetic Neuropathy	121 <sup>b</sup>	95 <sup>c</sup>	89 <sup>c</sup>	124 <sup>b</sup>

<sup>a</sup> = grains per unit area    <sup>b</sup> = p <0.05    <sup>c</sup> = p <0.01

25

These results confirm the *in vitro* observations in mesangial cells that there is a decrease of the  $\alpha 1$  integrin subunit and a concomitant increase of  $\alpha 2$  integrin

expression in a diabetic nephropathy. This represents a reversal of mesangial integrins which mediate binding of mesangial cells to collagen IV.

**Example 7**

**5        Increased Integrin Subunit Expression in Skin Fibroblasts  
From Diabetic Patients with Nephropathy  
as Compared with Control Diabetic Patients**

Fibroblasts were obtained from skin biopsies from diabetic patients with or without diabetic nephropathy and cultured as described for Example 6. Expression of 10  $\alpha_3$ ,  $\alpha_5$ , and beta-1 integrin subunits in the cultured cells was analyzed by Northern blotting and subsequent densitometry, as described above, and using published probes.

For the  $\alpha_3$  integrin subunit, the 1.9 SalI fragment described in Takada Y., et al., *J. Cell Biol.* 115:257-266 was used. For the  $\beta_1$  subunit, the 3.6 kb insert of the  $\beta_1$  subunit (the whole cDNA), described in Giancotti and Ruoslahti, *Cell* 60:849-850 15 (1990) was used. For the  $\alpha_5$  subunit, the 3.7 kb SalI-Xba insert of the  $\alpha_5$  subunit (the whole cDNA) described in Giancotti and Ruoslahti, *Supra* as used. These probes were radiolabeled and used under the same conditions as those described for Example 6.

The study included five patients per group, five each from the normal, diabetic "slow track" and from the Diabetic "fast track". Both groups of diabetic human subjects 20 had renal function studies and kidney biopsies performed as part of their evaluation as possible candidates for pancreas transplantation. All procedures were approved by the Committee on Human Subjects at the University of Minnesota, and all patients gave written consent. All patients spent one week at the Clinical Research Center (CRC) at the University of Minnesota for pre-pancreas transplant evaluation, during which time 25 they underwent multiple 24-hour urine collections (at least three) for measurements of creatinine clearance and urinary albumin excretion. Blood pressure was measured repeatedly by the CRC nursing staff. HbA1 was used to assess glycemic control. All patients underwent percutaneous kidney biopsy and skin biopsy. Patients were divided into two groups based on criteria of severity of renal lesions determined by 30 morphometric analysis of mesangial functional volume and IDDM duration.

"Normal" samples were kidney biopsies from non-diabetic human subjects, taken to examine for the presence of neoplastic tissue, etc., on which a similar analysis to that performed for the diabetic tissues was done. These subjects underwent similar

renal functional studies to make certain that albuminuria, increased creatinine clearance, or hypertension were not present.

The data, shown below in Table 3, demonstrate a significant increase in  $\alpha 3$  and beta-1 subunit expression in the skin fibroblasts of diabetic nephropathy patients as compared with the control diabetic patients.

TABLE 3

Integrin Subunit	Normal Values	Control Diabetic	Nephropathy Diabetics	P
$\alpha 3$	11.5 (9.1-13.3)	10.1 (8.6-12.8)	17.1 (16.1-35.6)	<0.5
$\alpha 5$	36.2 (18.3-46.6)	38.7 (31.6-57.2)	30.3 (13.2-48.4)	
$\beta 1$	29.9 (24.0-33.4)	24.9 (17.4-30.9)	37.1 (24.2-74.6)	<0.5

While particular embodiments of the invention have been described in detail, it will be apparent to those skilled in the art, that these embodiments are exemplary rather than limiting, and the true scope of the invention is that defined in the following claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

- (i) APPLICANT: Regents of the University of Minnesota
- (ii) TITLE OF THE INVENTION: ANALYSIS OF ALPHA INTEGRINS FOR THE DIAGNOSIS OF DIABETIC NEPHROPATHY
- (iii) NUMBER OF SEQUENCES: 16
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Merchant & Gould
  - (B) STREET: 3100 Norwest Center  
90 South 7th Street
  - (C) CITY: Minneapolis
  - (D) STATE: MN
  - (E) COUNTRY: US
  - (F) ZIP: 55402
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Diskette
  - (B) COMPUTER: IBM Compatible
  - (C) OPERATING SYSTEM: DOS
  - (D) SOFTWARE: FastSEQ Version 1.5
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: Unknown
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Kettelberger, Denise
  - (B) REGISTRATION NUMBER: 33,924
  - (C) REFERENCE/DOCKET NUMBER: 600.314USWO
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 612-332-5300
  - (B) TELEFAX: 612-332-9081
  - (C) TELEX:

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3987 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 420...3959

(A) NAME/KEY: mat\_peptide

(B) LOCATION: 504

(D) OTHER INFORMATION:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGTATGGAGA GAAAGGTCGTT TAAAAAGGCA GATGTCCCTT TAAGGTTTGC TTTGCTGCTG	60
CCCGTGGACT TTAGCCTAAA CAGGGTCCCG CGAAGTTGGC TTTATTTGTC CATGTCCTCGG	120
ACACAGCCTG GGTAGCTGCC AGTGAGATT CAGGGACCGA GCGCGCAAAG GGGGGGGAAA	180
TGTGGCAATC CACTCTGGAT GTGAGACGCG TGGAGAGGGC TTAGCAGCAT TTGACCAAAA	240
CACAGGAAAT CACTCCTCCA CAGCTCCTGG GCGCAGCAGC GGCTGGGGCC ACTGCCGGAC	300
ACCCTCGGAG ACCACACGAG TGACCCAGAG CGCAAGTCGC CAGCGTCCCG GTTCTGCCTG	360
TTCCTGCCAG CTCCCTGCCCA CGAACCGGCA CGTAGCTGGT TCCAGCAGCC GCTCCAGCA	419
ATG GTC CCC AGG CGT CCT GCC AGC CTA GAG GTC ACT GTA GCC TGC ATA	467
Met Val Pro Arg Arg Pro Ala Ser Leu Glu Val Thr Val Ala Cys Ile	
-28 -25 -20 -15	
TGG CTT CTC ACG GTC ATC CTA GGC TTC TGC GTC TCC TTC AAT GTT GAT	515
Trp Leu Thr Val Ile Leu Gly Phe Cys Val Ser Phe Asn Val Asp	
-10 -5 1	
GTG AAA AAC TCA ATG AGT TTC AGT GGC CCA GTA GAG GAC ATG TTT GGA	563
Val Lys Asn Ser Met Ser Phe Ser Gly Pro Val Glu Asp Met Phe Gly	
5 10 15 20	
TAC ACT GTT CAA CAA TAT GAA AAC GAA GAA GGC AAA TGG GTT CTT ATT	611
Tyr Thr Val Gln Gln Tyr Glu Asn Glu Glu Gly Lys Trp Val Leu Ile	
25 30 35	
GGT TCT CCT TTA GTT GGC CAA CCC AAA GCA AGA ACT GGA GAT GTC TAT	659
Gly Ser Pro Leu Val Gly Gln Pro Lys Ala Arg Thr Gly Asp Val Tyr	
40 45 50	
AAG TGT CCG GTT GGG AGA GAG AGA GCA ATG CCT TGC GTG AAG TTG GAC	707
Lys Cys Pro Val Gly Arg Glu Arg Ala Met Pro Cys Val Lys Leu Asp	
55 60 65	

TTG CCA GTT AAC ACA TCG ATC CCC AAT GTC ACA GAA ATA AAG GAA AAC Glu Pro Val Asn Thr Ser Ile Pro Asn Val Thr Glu Ile Lys Glu Asn 70 75 80	755
ATG ACA TTT GGA TCA ACT TTA GTC ACC AAC CCG AAT GGA GGA TTT CTG Met Thr Phe Gly Ser Thr Leu Val Thr Asn Pro Asn Gly Gly Phe Leu 85 90 95 100	803
GCA TGT GGG CCC TTG TAT GCC TAT AGA TGT GGA CAT TTG CAT TAT ACA Ala Cys Gly Pro Leu Tyr Ala Tyr Arg Cys Gly His Leu His Tyr Thr 105 110 115	851
ACT GGA ATA TGT TCT GAT GTC AGT CCT ACA TTT CAA GTT GTG AAC TCC Thr Gly Ile Cys Ser Asp Val Ser Pro Thr Phe Gln Val Val Asn Ser 120 125 130	899
TTT GCC CCT GTA CAA GAA TGC AGC ACC CAG CTG GAC ATA GTC ATC GTC Phe Ala Pro Val Gln Glu Cys Ser Thr Gln Leu Asp Ile Val Ile Val 135 140 145	947
CTG GAT GGC TCC AAC AGC ATC TAC CCC TGG GAA AGT GTC ATC GCC TTT Leu Asp Gly Ser Asn Ser Ile Tyr Pro Trp Glu Ser Val Ile Ala Phe 150 155 160	995
TTA AAC GAC CTT CTT AAG AGG ATG GAT ATT GGC CCT AAG CAG ACA CAG Leu Asn Asp Leu Leu Lys Arg Met Asp Ile Gly Pro Lys Gln Thr Gln 165 170 175 180	1043
GTC GGG ATT GTA CAG TAT GGA GAG AAT GTA ACC CAT GAG TTC AAC CTC Val Gly Ile Val Gln Tyr Gly Glu Asn Val Thr His Glu Phe Asn Leu 185 190 195	1091
AAT AAG TAT TCA TCC ACA GAA GAG GTC CTT GTC GCA GCA AAC AAA ATA Asn Lys Tyr Ser Ser Thr Glu Glu Val Leu Val Ala Ala Asn Lys Ile 200 205 210	1139
GGC CGA CAG GGA GGC CTC CAA ACG ATG ACA GCC CTT GGA ATA GAC ACA Gly Arg Gln Gly Leu Gln Thr Met Thr Ala Leu Gly Ile Asp Thr 215 220 225	1187
GCC AGG AAA GAG GCA TTC ACT GAA GCT CGG GGT GCC AGG AGG GGA GTT Ala Arg Lys Glu Ala Phe Thr Glu Ala Arg Gly Ala Arg Arg Gly Val 230 235 240	1235
AAA AAA GTC ATG GTT ATT GTG ACC GAC GGA GAA TCG CAT GAC AAC TAT Lys Lys Val Met Val Ile Val Thr Asp Gly Glu Ser His Asp Asn Tyr 245 250 255 260	1283
CGC TGA AAC AGG TCA TCC AAG ACT GCG AGG ACG AAA ACA TTC AGC GAT Arg Leu Lys Gln Val Ile Gln Asp Cys Glu Asp Glu Asn Ile Gln Arg 265 270 275	1331
TTT TCC ATA GCT ATC CTT GGC CAC TAT AAC AGG GGG AAC TTA AGC ACT Phe Ser Ile Ala Ile Leu Gly His Tyr Asn Arg Gly Asn Leu Ser Thr 280 285 290	1379

GAA AAA TTT GTG GAG GAA ATA AAA TCG ATC GCA AGC GAG CCC ACG GAA Glu Lys Phe Val Glu Glu Ile Lys Ser Ile Ala Ser Glu Pro Thr Glu 295	300	305	1427
AAG CAC TTC TTC AAT GTC TCG GAT GAG TTG GCC CTG GTC ACT ATT GTT Lys His Phe Phe Asn Val Ser Asp Glu Leu Ala Leu Val Thr Ile Val 310	315	320	1475
AAA GCT CTG GGA GAA AGG ATA TTC GCT TTG GAA GCG ACA GCT GAC CAG Lys Ala Leu Gly Glu Arg Ile Phe Ala Leu Glu Ala Thr Ala Asp Gln 325	330	335	1523
TCA GCA GCT TCA TTT GAG ATG GAA ATG TCT CAG ACT GGC TTC AGT GCT Ser Ala Ala Ser Phe Glu Met Glu Met Ser Gln Thr Gly Phe Ser Ala 345	350	355	1571
CAC TAC TCC CAG GAC TGG GTC ATG CTT GGA GCG GTG GGA GCC TAT GAC His Tyr Ser Gln Asp Trp Val Met Leu Gly Ala Val Gly Ala Tyr Asp 360	365	370	1619
TGG AAC GGA ACT GTG GTC ATG CAG AAG GCT AAC CAG ATG GTC ATC CCT Trp Asn Gly Thr Val Val Met Gln Lys Ala Asn Gln Met Val Ile Pro 375	380	385	1667
CAT AAC ACC ACC TTT CAA ACT GAG CCC GCC AAG ATG AAC GAG CCT CTG His Asn Thr Thr Phe Gln Thr Glu Pro Ala Lys Met Asn Glu Pro Leu 390	395	400	1715
GCT TCT TAT TTA GGT TAC ACA GTG AAC TCG GCC ACC ATC CCT GGA GAT Ala Ser Tyr Leu Gly Tyr Thr Val Asn Ser Ala Thr Ile Pro Gly Asp 405	410	415	1763
GTG CTC TAC ATC GCT GGG CAG CCT CGG TAC AAT CAT ACG GGC CAG GTC Val Leu Tyr Ile Ala Gly Gln Pro Arg Tyr Asn His Thr Gly Gln Val 425	430	435	1811
GTC ATC TAC AAG ATG GAG GAT GGG AAC ATC AAC ATT CTG CAG ACA CTC Val Ile Tyr Lys Met Glu Asp Gly Asn Ile Asn Ile Leu Gln Thr Leu 440	445	450	1859
GGC GGA GAG CAG ATT GGT TCC TAC TTT GGT AGT GTC TTA ACA ACA ATT Gly Gly Glu Gln Ile Gly Ser Tyr Phe Gly Ser Val Leu Thr Thr Ile 455	460	465	1907
GAC ATC GAC AAA GAT TCT TAT ACT GAT CTG CTT CTC GTC GGG GCC CCC Asp Ile Asp Lys Asp Ser Tyr Thr Asp Leu Leu Leu Val Gly Ala Pro 470	475	480	1955
ATG TAC ATG GGG ACA GAG AAA GAG GAA CAG GGC AAG GTG TAC GTG TAC Met Tyr Met Gly Thr Glu Lys Glu Glu Gln Gly Lys Val Tyr Val Tyr 485	490	495	2003
GCT GTG AAT CAG ACA AGG TTT GAA TAT CAA ATG AGC CTG GAA CCA ATT Ala Val Asn Gln Thr Arg Phe Glu Tyr Gln Met Ser Leu Glu Pro Ile 505	510	515	2051

GGC AGA CCT GCT CAT CCC TGA AGG ATA ATT CAT GCA CGA AAG AAA Arg Gln Thr Cys Cys Ser Ser Leu Lys Asp Asn Ser Cys Thr Lys Glu 520 525 530	2099
AAC AAG AAT GAG CCC TGC GGG GCC CGC TTC GGA ACA GCA ATT GCT GCT Asn Lys Asn Glu Pro Cys Gly Ala Arg Phe Gly Thr Ala Ile Ala Ala 535 540 545	2147
GTA AAA GAC CTC AAC GTG GAT GGA TTT AAT GAC GTC GTG ATT GGA GCT Val Lys Asp Leu Asn Val Asp Gly Phe Asn Asp Val Val Ile Gly Ala 550 555 560	2195
CCG CTG GAA GAT GAC CAC GCA GGA GCT GTG TAC ATT TAT CAT GGC AGT Pro Leu Glu Asp Asp His Ala Gly Ala Val Tyr Ile Tyr His Gly Ser 565 570 575 580	2243
GGC AAG ACC ATA AGG GAG GCG TAT GCA CAA CGC ATT CCA TCA GGT GGG Gly Lys Thr Ile Arg Glu Ala Tyr Ala Gln Arg Ile Pro Ser Gly Gly 585 590 595	2291
GAT GGC AAG ACC CTG AAA TTT TTC GGC CAG TCT ATC CAC GGA GAG ATG Asp Gly Lys Thr Leu Lys Phe Phe Gly Gln Ser Ile His Gly Glu Met 600 605 610	2339
GAT TTA AAT GGT GAC GGT CTG ACT GAC GTG ACC ATT GGA GGC CTT GGT Asp Leu Asn Gly Asp Gly Leu Thr Asp Val Thr Ile Gly Gly Leu Gly 615 620 625	2387
GGA GCA GCC CTC TTC TGG GCC AGA GAT GTG GCT GTA GTT AAA GTG ACC Gly Ala Ala Leu Phe Trp Ala Arg Asp Val Ala Val Val Lys Val Thr 630 635 640	2435
ATG AAT TTT GAA CCC AAT AAA GTG AAT ATT CAA AAG AAA AAC TGC CGT Met Asn Phe Glu Pro Asn Lys Val Asn Ile Gln Lys Lys Asn Cys Arg 645 650 655 660	2483
GTG GAG GGC AAA GAA ACA GTG TGC ATA AAT GCT ACA ATG TGT TTT CAT Val Glu Gly Lys Glu Thr Val Cys Ile Asn Ala Thr Met Cys Phe His 665 670 675	2531
GTG AAA TTA AAG TCT AAA GAG GAC TCA ATT TAC GAG GCT GAT CTG CAG Val Lys Leu Lys Ser Lys Glu Asp Ser Ile Tyr Glu Ala Asp Leu Gln 680 685 690	2579
TAC CGT GTC ACC CTT GAT TCA CTG AGG CAG ATA TCA CGG AGC TTT TTT Tyr Arg Val Thr Leu Asp Ser Leu Arg Gln Ile Ser Arg Ser Phe Phe 695 700 705	2627
TCT GGA ACT CAG GAA AGG AAG ATT CAA AGA AAT ATC ACC GTT CGA GAA Ser Gly Thr Gln Glu Arg Lys Ile Gln Arg Asn Ile Thr Val Arg Glu 710 715 720	2675
TCA GAA TGC ATC AGG CAC TCC TTC TAC ATG TTG GAC AAA CAT GAC TTT Ser Glu Cys Ile Arg His Ser Phe Tyr Met Leu Asp Lys His Asp Phe 725 730 735 740	2723

CAG GAC TCT GTG AGA GTG ACT CTG GAT TTT AAT CTC ACT GAT CCA GAA Gln Asp Ser Val Arg Val Thr Leu Asp Phe Asn Leu Thr Asp Pro Glu 745 750 755	2771
AAT GGT CCT GTA CTT GAT GAC GCT CTG CCA AAC TCA GTC CAC GAA CAC Asn Gly Pro Val Leu Asp Asp Ala Leu Pro Asn Ser Val His Glu His 760 765 770	2819
ATT CCC TTT GCC AAA GAC TGT GGA AAC AAG GAA AGA TGC ATT TCA GAC Ile Pro Phe Ala Lys Asp Cys Gly Asn Lys Glu Arg Cys Ile Ser Asp 775 780 785	2867
CTC ACT CTG AAT GTG TCC ACC ACA GAA AAG AGC CTG CTG ATC GTC AAG Leu Thr Leu Asn Val Ser Thr Thr Glu Lys Ser Leu Leu Ile Val Lys 790 795 800	2915
TCC CAG CAT GAC AAG TTC AAC GTT AGC CTC ACC GTC AAA AAC AAA GGA Ser Gln His Asp Lys Phe Asn Val Ser Leu Thr Val Lys Asn Lys Gly 805 810 815 820	2963
GAC AGT GCG TAC AAC ACC AGG ACA GTG GTG CAG CAT TCA CCA AAT CTG Asp Ser Ala Tyr Asn Thr Arg Thr Val Val Gln His Ser Pro Asn Leu 825 830 835	3011
ATT TTT TCG GGA ATT GAG GAG ATC CAA AAA GAT AGC TGT GAA TCT AAT Ile Phe Ser Gly Ile Glu Glu Ile Gln Lys Asp Ser Cys Glu Ser Asn 840 845 850	3059
CAA AAT ATC ACT TGC AGA GTT GGA TAT CCT TTC CTA AGA GCA GGA GAA Gln Asn Ile Thr Cys Arg Val Gly Tyr Pro Phe Leu Arg Ala Gly Glu 855 860 865	3107
ACG GTT ACC TTC AAA ATA ATA TTC CAG TTT AAC ACA TCC CAT CTC TCG Thr Val Thr Phe Lys Ile Ile Phe Gln Phe Asn Thr Ser His Leu Ser 870 875 880	3155
GAA AAT GCA ATC ATT CAC TTA AGT GCA ACA AGT GAC AGT GAG GAG CCC Glu Asn Ala Ile Ile His Leu Ser Ala Thr Ser Asp Ser Glu Glu Pro 885 890 895 900	3203
CTG GAA TCT CTT AAT GAT AAT GAA GTA AAT ATT TCC ATC CCA GTA AAA Leu Glu Ser Leu Asn Asp Asn Glu Val Asn Ile Ser Ile Pro Val Lys 905 910 915	3251
TAT GAA GTT GGA CTG CAG TTT TAC AGT TCT GCG AGT GAA CAT CAC ATT Tyr Glu Val Gly Leu Gln Phe Tyr Ser Ser Ala Ser Glu His His Ile 920 925 930	3299
TCA GTC GCT GCC AAT GAG ACG ATC CCT GAG TTT ATT AAC TCC ACT GAG Ser Val Ala Ala Asn Glu Thr Ile Pro Glu Phe Ile Asn Ser Thr Glu 935 940 945	3347
GAC ATT GGG AAT GAA ATT AAT GTC TTC TAT ACG ATT AGA AAG AGG GGG Asp Ile Gly Asn Glu Ile Asn Val Phe Tyr Thr Ile Arg Lys Arg Gly 950 955 960	3395

CAT TTC CCA ATG CCA GAA CTT CAG CTG TCA ATT TCA TTC CCC AAT TTG His Phe Pro Met Pro Glu Leu Gln Leu Ser Ile Ser Phe Pro Asn Leu 965 970 975 980	3443
ACG GCA GAT GGT TAT CCT GTA CTG TAC CCA ATT GGA TGG TCA TCT TCA Thr Ala Asp Gly Tyr Pro Val Leu Tyr Pro Ile Gly Trp Ser Ser Ser 985 990 995	3491
GAT AAT GTG AAC TGT AGA CCC CGG AGC CTT GAG GAC CCC TTT GGC ATC Asp Asn Val Asn Cys Arg Pro Arg Ser Leu Glu Asp Pro Phe Gly Ile 1000 1005 1010	3539
AAC TCT GGG AAG AAA ATG ACA ATA TCG AAG TCT GAG GTT CTC AAA AGA Asn Ser Gly Lys Lys Met Thr Ile Ser Lys Ser Glu Val Leu Lys Arg 1015 1020 1025	3587
GGC ACA ATC CAG GAC TGC AGT AGT ACG TGT GGA GTT GCC ACC ATC ACG Gly Thr Ile Gln Asp Cys Ser Ser Thr Cys Gly Val Ala Thr Ile Thr 1030 1035 1040	3635
TGT AGC CTC CTT CCT TCC GAC CTG AGT CAA GTG AAT GTC TCG CTC CTC Cys Ser Leu Leu Pro Ser Asp Leu Ser Gln Val Asn Val Ser Leu Leu 1045 1050 1055 1060	3683
CTG TGG AAA CCG ACT TTC ATA AGA GCA CAT TTT TCC AGC TTA AAC CTT Leu Trp Lys Pro Thr Phe Ile Arg Ala His Phe Ser Ser Leu Asn Leu 1065 1070 1075	3731
ACT CTA AGA GGA GAA CTT AAG AGT GAA AAT TCA TCG CTG ACT TTA AGT Thr Leu Arg Gly Glu Leu Lys Ser Glu Asn Ser Ser Leu Thr Leu Ser 1080 1085 1090	3779
AGC AGC AAC CGG AAG CGA GAG CTG GCT ATT CAG ATA TCC AAA GAC GGG Ser Ser Asn Arg Lys Arg Glu Leu Ala Ile Gln Ile Ser Lys Asp Gly 1095 1100 1105	3827
CTC CCA GGC AGA GTG CCG CTG TGG GTT ATC CTC CTG AGC GCC TTC GCG Leu Pro Gly Arg Val Pro Leu Trp Val Ile Leu Leu Ser Ala Phe Ala 1110 1115 1120	3875
GGG CTA CTG CTG CTA ATG CTC CTT ATA TTG GCT CTG TGG AAG ATT GGA Gly Leu Leu Leu Leu Met Leu Leu Ile Leu Ala Leu Trp Lys Ile Gly 1125 1130 1135 1140	3923
TTC TTC AAA AGG CCA CTG AAG AAG AAA ATG GAG AAA TGAAAGGTTT Phe Phe Lys Arg Pro Leu Lys Lys Lys Met Glu Lys 1145 1150	3969
<b>CATAGAAAAA AAAAAAAAAA</b>	<b>3987</b>

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1180 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Val Pro Arg Arg Pro Ala Ser Leu Glu Val Thr Val Ala Cys Ile				
-28	-25	-20		-15

Trp Leu Leu Thr Val Ile Leu Gly Phe Cys Val Ser Phe Asn Val Asp				
-10	-5		1	

Val Lys Asn Ser Met Ser Phe Ser Gly Pro Val Glu Asp Met Phe Gly				
5	10	15		20

Tyr Thr Val Gln Gln Tyr Glu Asn Glu Glu Gly Lys Trp Val Leu Ile				
25	30		35	

Gly Ser Pro Leu Val Gly Gln Pro Lys Ala Arg Thr Gly Asp Val Tyr				
40	45		50	

Lys Cys Pro Val Gly Arg Glu Arg Ala Met Pro Cys Val Lys Leu Asp				
55	60		65	

Glu Pro Val Asn Thr Ser Ile Pro Asn Val Thr Glu Ile Lys Glu Asn				
70	75		80	

Met Thr Phe Gly Ser Thr Leu Val Thr Asn Pro Asn Gly Gly Phe Leu				
85	90	95		100

Ala Cys Gly Pro Leu Tyr Ala Tyr Arg Cys Gly His Leu His Tyr Thr				
105	110		115	

Thr Gly Ile Cys Ser Asp Val Ser Pro Thr Phe Gln Val Val Asn Ser				
120	125		130	

Phe Ala Pro Val Gln Glu Cys Ser Thr Gln Leu Asp Ile Val Ile Val				
135	140		145	

Leu Asp Gly Ser Asn Ser Ile Tyr Pro Trp Glu Ser Val Ile Ala Phe				
150	155		160	

Leu Asn Asp Leu Leu Lys Arg Met Asp Ile Gly Pro Lys Gln Thr Gln				
165	170	175		180

Val Gly Ile Val Gln Tyr Gly Glu Asn Val Thr His Glu Phe Asn Leu				
185	190		195	

Asn Lys Tyr Ser Ser Thr Glu Glu Val Leu Val Ala Ala Asn Lys Ile  
200 205 210

Gly Arg Gln Gly Gly Leu Gln Thr Met Thr Ala Leu Gly Ile Asp Thr  
215 220 225

Ala Arg Lys Glu Ala Phe Thr Glu Ala Arg Gly Ala Arg Arg Gly Val  
230 235 240

Lys Lys Val Met Val Ile Val Thr Asp Gly Glu Ser His Asp Asn Tyr  
245 250 255 260

Arg Leu Lys Gln Val Ile Gln Asp Cys Glu Asp Glu Asn Ile Gln Arg  
265 270 275

Phe Ser Ile Ala Ile Leu Gly His Tyr Asn Arg Gly Asn Leu Ser Thr  
280 285 290

Glu Lys Phe Val Glu Glu Ile Lys Ser Ile Ala Ser Glu Pro Thr Glu  
295 300 305

Lys His Phe Phe Asn Val Ser Asp Glu Leu Ala Leu Val Thr Ile Val  
310 315 320

Lys Ala Leu Gly Glu Arg Ile Phe Ala Leu Glu Ala Thr Ala Asp Gln  
325 330 335 340

Ser Ala Ala Ser Phe Glu Met Glu Met Ser Gln Thr Gly Phe Ser Ala  
345 350 355

His Tyr Ser Gln Asp Trp Val Met Leu Gly Ala Val Gly Ala Tyr Asp  
360 365 370

Trp Asn Gly Thr Val Val Met Gln Lys Ala Asn Gln Met Val Ile Pro  
375 380 385

His Asn Thr Thr Phe Gln Thr Glu Pro Ala Lys Met Asn Glu Pro Leu  
390 395 400

Ala Ser Tyr Leu Gly Tyr Thr Val Asn Ser Ala Thr Ile Pro Gly Asp  
405 410 415 420

Val Leu Tyr Ile Ala Gly Gln Pro Arg Tyr Asn His Thr Gly Gln Val  
425 430 435

Val Ile Tyr Lys Met Glu Asp Gly Asn Ile Asn Ile Leu Gln Thr Leu  
440 445 450

Gly Gly Glu Gln Ile Gly Ser Tyr Phe Gly Ser Val Leu Thr Thr Ile  
455 460 465

Asp Ile Asp Lys Asp Ser Tyr Thr Asp Leu Leu Leu Val Gly Ala Pro  
470 475 480

Met Tyr Met Gly Thr Glu Lys Glu Glu Gln Gly Lys Val Tyr Val Tyr  
485 490 495 500

Ala Val Asn Gln Thr Arg Phe Glu Tyr Gln Met Ser Leu Glu Pro Ile  
 505 510 515  
 Arg Gln Thr Cys Cys Ser Ser Leu Lys Asp Asn Ser Cys Thr Lys Glu  
 520 525 530  
 Asn Lys Asn Glu Pro Cys Gly Ala Arg Phe Gly Thr Ala Ile Ala Ala  
 535 540 545  
 Val Lys Asp Leu Asn Val Asp Gly Phe Asn Asp Val Val Ile Gly Ala  
 550 555 560  
 Pro Leu Glu Asp Asp His Ala Gly Ala Val Tyr Ile Tyr His Gly Ser  
 565 570 575 580  
 Gly Lys Thr Ile Arg Glu Ala Tyr Ala Gln Arg Ile Pro Ser Gly Gly  
 585 590 595  
 Asp Gly Lys Thr Leu Lys Phe Phe Gly Gln Ser Ile His Gly Glu Met  
 600 605 610  
 Asp Leu Asn Gly Asp Gly Leu Thr Asp Val Thr Ile Gly Gly Leu Gly  
 615 620 625  
 Gly Ala Ala Leu Phe Trp Ala Arg Asp Val Ala Val Val Lys Val Thr  
 630 635 640  
 Met Asn Phe Glu Pro Asn Lys Val Asn Ile Gln Lys Lys Asn Cys Arg  
 645 650 655 660  
 Val Glu Gly Lys Glu Thr Val Cys Ile Asn Ala Thr Met Cys Phe His  
 665 670 675  
 Val Lys Leu Lys Ser Lys Glu Asp Ser Ile Tyr Glu Ala Asp Leu Gln  
 680 685 690  
 Tyr Arg Val Thr Leu Asp Ser Leu Arg Gln Ile Ser Arg Ser Phe Phe  
 695 700 705  
 Ser Gly Thr Gln Glu Arg Lys Ile Gln Arg Asn Ile Thr Val Arg Glu  
 710 715 720  
 Ser Glu Cys Ile Arg His Ser Phe Tyr Met Leu Asp Lys His Asp Phe  
 725 730 735 740  
 Gln Asp Ser Val Arg Val Thr Leu Asp Phe Asn Leu Thr Asp Pro Glu  
 745 750 755  
 Asn Gly Pro Val Leu Asp Asp Ala Leu Pro Asn Ser Val His Glu His  
 760 765 770  
 Ile Pro Phe Ala Lys Asp Cys Gly Asn Lys Glu Arg Cys Ile Ser Asp  
 775 780 785  
 Leu Thr Leu Asn Val Ser Thr Thr Glu Lys Ser Leu Leu Ile Val Lys  
 790 795 800

Ser Gln His Asp Lys Phe Asn Val Ser Leu Thr Val Lys Asn Lys Gly  
805 810 815 820

Asp Ser Ala Tyr Asn Thr Arg Thr Val Val Gln His Ser Pro Asn Leu  
825 830 835

Ile Phe Ser Gly Ile Glu Glu Ile Gln Lys Asp Ser Cys Glu Ser Asn  
840 845 850

Gln Asn Ile Thr Cys Arg Val Gly Tyr Pro Phe Leu Arg Ala Gly Glu  
855 860 865

Thr Val Thr Phe Lys Ile Ile Phe Gln Phe Asn Thr Ser His Leu Ser  
870 875 880

Glu Asn Ala Ile Ile His Leu Ser Ala Thr Ser Asp Ser Glu Glu Pro  
885 890 895 900

Leu Glu Ser Leu Asn Asp Asn Glu Val Asn Ile Ser Ile Pro Val Lys  
905 910 915

Tyr Glu Val Gly Leu Gln Phe Tyr Ser Ser Ala Ser Glu His His Ile  
920 925 930

Ser Val Ala Ala Asn Glu Thr Ile Pro Glu Phe Ile Asn Ser Thr Glu  
935 940 945

Asp Ile Gly Asn Glu Ile Asn Val Phe Tyr Thr Ile Arg Lys Arg Gly  
950 955 960

His Phe Pro Met Pro Glu Leu Gln Leu Ser Ile Ser Phe Pro Asn Leu  
965 970 975 980

Thr Ala Asp Gly Tyr Pro Val Leu Tyr Pro Ile Gly Trp Ser Ser Ser  
985 990 995

Asp Asn Val Asn Cys Arg Pro Arg Ser Leu Glu Asp Pro Phe Gly Ile  
1000 1005 1010

Asn Ser Gly Lys Lys Met Thr Ile Ser Lys Ser Glu Val Leu Lys Arg  
1015 1020 1025

Gly Thr Ile Gln Asp Cys Ser Ser Thr Cys Gly Val Ala Thr Ile Thr  
1030 1035 1040

Cys Ser Leu Leu Pro Ser Asp Leu Ser Gln Val Asn Val Ser Leu Leu  
1045 1050 1055 1060

Leu Trp Lys Pro Thr Phe Ile Arg Ala His Phe Ser Ser Leu Asn Leu  
1065 1070 1075

Thr Leu Arg Gly Glu Leu Lys Ser Glu Asn Ser Ser Leu Thr Leu Ser  
1080 1085 1090

Ser Ser Asn Arg Lys Arg Glu Leu Ala Ile Gln Ile Ser Lys Asp Gly  
1095 1100 1105

Leu Pro Gly Arg Val Pro Leu Trp Val Ile Leu Leu Ser Ala Phe Ala  
1110 1115 1120

Gly Leu Leu Leu Leu Met Leu Leu Ile Leu Ala Leu Trp Lys Ile Gly  
1125 1130 1135 1140

Phe Phe Lys Arg Pro Leu Lys Lys Lys Met Glu Lys  
1145 1150

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5373 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO
  - (iv) ANTISENSE: NO
  - (v) FRAGMENT TYPE:
  - (vi) ORIGINAL SOURCE:
  - (ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 49...3591
- (D) OTHER INFORMATION:

- (A) NAME/KEY: mat\_peptide
- (B) LOCATION: 136
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3

**GAATTCTGC AAACCCAGCG CAACTACGGT CCCCCGGTCA GACCCAGG ATG GGG CCA**  
Met Gly Pro  
-29

GAA CGG ACA GGG GCC GCG CCG CTG CCG CTG CTG CTG GTG TTA GCG CTC 105  
 Glu Arg Thr Gly Ala Ala Pro Leu Pro Leu Leu Leu Val Leu Ala Leu  
 -25 -20 -15

AGT CAA GGC ATT TTA AAT TGT TGT TTG GCC TAC AAT GTT GGT CTC CCA 153  
 Ser Gln Gly Ile Leu Asn Cys Cys Leu Ala Tyr Asn Val Gly Leu Pro  
 -10 -5 1 5

GAA GCA AAA ATA TTT TCC GGT CCT TCA AGT GAA CAG TTT GGG TAT GCA      201  
 Glu Ala Lys Ile Phe Ser Gly Pro Ser Ser Glu Gln Phe Gly Tyr Ala  
           10            15            20

GTC CAG CAG TTT ATA AAT CCA AAA GGC AAC TGG TTA CTG GTT GGT TCA 249  
 Val Gln Gln Phe Ile Asn Pro Lys Gly Asn Trp Leu Leu Val Gly Ser  
           25                 30                 35

CCC TGG AGT GGC TTT CCT GAG AAC CGA ATG GGA GAT GTG TAT AAA TGT Pro Trp Ser Gly Phe Pro Glu Asn Arg Met Gly Asp Val Tyr Lys Cys 40 45 50	297
CCT GTT GAC CTA TCC ACT GCC ACA TGT GAA AAA CTA AAT TTG CAA ACT Pro Val Asp Leu Ser Thr Ala Thr Cys Glu Lys Leu Asn Leu Gln Thr 55 60 65 70	345
TCA ACA AGC ATT CCA AAT GTT ACT GAG ATG AAA ACC AAC ATG AGC CTC Ser Thr Ser Ile Pro Asn Val Thr Glu Met Lys Thr Asn Met Ser Leu 75 80 85	393
GGC TTG ATC CTC ACC AGG AAC ATG GGA ACT GGA GGT TTT CTC ACA TGT Gly Leu Ile Leu Thr Arg Asn Met Gly Thr Gly Gly Phe Leu Thr Cys 90 95 100	441
GGT CCT CTG TGG GCA CAG CAA TGT GGG AAT CAG TAT TAC ACA ACG GGT Gly Pro Leu Trp Ala Gln Gln Cys Gly Asn Gln Tyr Tyr Thr Thr Gly 105 110 115	489
GTG TGT TCT GAC ATC AGT CCT GAT TTT CAG CTC TCA GCC AGC TTC TCA Val Cys Ser Asp Ile Ser Pro Asp Phe Gln Leu Ser Ala Ser Phe Ser 120 125 130	537
CCT GCA ACT CAG CCC TGC CCT TCC CTC ATA GAT GTT GTG GTT GTG TGT Pro Ala Thr Gln Pro Cys Pro Ser Leu Ile Asp Val Val Val Val Cys 135 140 145 150	585
GAT GAA TCA AAT AGT ATT TAT CCT TGG GAT GCA GTA AAG AAT TTT TTG Asp Glu Ser Asn Ser Ile Tyr Pro Trp Asp Ala Val Lys Asn Phe Leu 155 160 165	633
GAA AAA TTT GTA CAA GGC CTT GAT ATA GGC CCC ACA AAG ACA CAG GTG Glu Lys Phe Val Gln Gly Leu Asp Ile Gly Pro Thr Lys Thr Gln Val 170 175 180	681
GGG TTA ATT CAG TAT GCC AAT AAT CCA AGA GTT GTG TTT AAC TTG AAC Gly Leu Ile Gln Tyr Ala Asn Asn Pro Arg Val Val Phe Asn Leu Asn 185 190 195	729
ACA TAT AAA ACC AAA GAA GAA ATG ATT GTA GCA ACA TCC CAG ACA TCC Thr Tyr Lys Thr Lys Glu Glu Met Ile Val Ala Thr Ser Gln Thr Ser 200 205 210	777
CAA TAT GGT GGG GAC CTC ACA AAC ACA TTC GGA GCA ATT CAA TAT GCA Gln Tyr Gly Gly Asp Leu Thr Asn Thr Phe Gly Ala Ile Gln Tyr Ala 215 220 225 230	825
AGA AAA TAT GCC TAT TCA GCA GCT TCT GGT GGG CGA CGA AGT GCT ACG Arg Lys Tyr Ala Tyr Ser Ala Ala Ser Gly Gly Arg Arg Ser Ala Thr 235 240 245	873
AAA GTA ATG GTA GTT GTA ACT GAC GGT GAA TCA CAT GAT GGT TCA ATG Lys Val Met Val Val Thr Asp Gly Glu Ser His Asp Gly Ser Met 250 255 260	921

TTG AAA GCT GTG ATT GAT CAA TGC AAC CAT GAC AAT ATA CTG AGG TTT Leu Lys Ala Val Ile Asp Gln Cys Asn His Asp Asn Ile Leu Arg Phe 265	270	275	969
GGC ATA GCA GTT CTT GGG TAC TTA AAC AGA AAC GCC CTT GAT ACT AAA Gly Ile Ala Val Leu Gly Tyr Leu Asn Arg Asn Ala Leu Asp Thr Lys 280	285	290	1017
AAT TTA ATA AAA GAA ATA AAA GCG ATC GCT AGT ATT CCA ACA GAA AGA Asn Leu Ile Lys Glu Ile Lys Ala Ile Ala Ser Ile Pro Thr Glu Arg 295	300	305	1065
TAC TTT TTC AAT GTG TCT GAT GAA GCA GCT CTA CTA GAA AAG GCT GGG Tyr Phe Phe Asn Val Ser Asp Glu Ala Ala Leu Leu Glu Lys Ala Gly 315	320	325	1113
ACA TTA GGA GAA CAA ATT TTC AGC ATT GAA GGT ACT GTT CAA GGA GGA Thr Leu Gly Glu Gln Ile Phe Ser Ile Glu Gly Thr Val Gln Gly Gly 330	335	340	1161
GAC AAC TTT CAG ATG GAA ATG TCA CAA GTG GGA TTC AGT GCA GAT TAC Asp Asn Phe Gln Met Glu Met Ser Gln Val Gly Phe Ser Ala Asp Tyr 345	350	355	1209
TCT TCT CAA AAT GAT ATT CTG ATG CTG GGT GCA GTG GGA GCT TTT GGC Ser Ser Gln Asn Asp Ile Leu Met Leu Gly Ala Val Gly Ala Phe Gly 360	365	370	1257
TGG AGT GGG ACC ATT GTC CAG AAG ACA TCT CAT GGC CAT TTG ATC TTT Trp Ser Gly Thr Ile Val Gln Lys Thr Ser His Gly His Leu Ile Phe 375	380	385	1305
CCT AAA CAA GCC TTT GAC CAA ATT CTG CAG GAC AGA AAT CAC AGT TCA Pro Lys Gln Ala Phe Asp Gln Ile Leu Gln Asp Arg Asn His Ser Ser 395	400	405	1353
TAT TTA GGT TAC TCT GTG GCT GCA ATT TCT ACT GGA GAA AGC ACT CAC Tyr Leu Gly Tyr Ser Val Ala Ala Ile Ser Thr Gly Glu Ser Thr His 410	415	420	1401
TTT GTT GCT GGT GCT CCT CGG GCA AAT TAT ACC GGC CAG ATA GTG CTA Phe Val Ala Gly Ala Pro Arg Ala Asn Tyr Thr Gly Gln Ile Val Leu 425	430	435	1449
TAT AGT GTG AAT GAG AAT GGC AAT ATC ACG GTT ATT CAG GCT CAC CGA Tyr Ser Val Asn Glu Asn Gly Asn Ile Thr Val Ile Gln Ala His Arg 440	445	450	1497
GGT GAC CAG ATT GGC TCC TAT TTT GGT AGT GTG CTG TGT TCA GTT GAT Gly Asp Gln Ile Gly Ser Tyr Phe Gly Ser Val Leu Cys Ser Val Asp 455	460	465	1545
GTG GAT AAA GAC ACC ATT ACA GAC GTG CTC TTG GTA GGT GCA CCA ATG Val Asp Lys Asp Thr Ile Thr Asp Val Leu Leu Val Gly Ala Pro Met 475	480	485	1593

TAC ATG AGT GAC CTA AAG AAA GAG GAA GGA AGA GTC TAC CTG TTT ACT Tyr Met Ser Asp Leu Lys Lys Glu Glu Gly Arg Val Tyr Leu Phe Thr 490 495 500	1641
ATC AAA AAG GGC ATT TTG GGT CAG CAC CAA TTT CTT GAA GGC CCC GAG Ile Lys Lys Gly Ile Leu Gly Gln His Gln Phe Leu Glu Gly Pro Glu 505 510 515	1689
GGC ATT GAA AAC ACT CGA TTT GGT TCA GCA ATT GCA GCT CTT TCA GAC Gly Ile Glu Asn Thr Arg Phe Gly Ser Ala Ile Ala Ala Leu Ser Asp 520 525 530	1737
ATC AAC ATG GAT GGC TTT AAT GAT GTG ATT GTT GGT TCA CCA CTA GAA Ile Asn Met Asp Gly Phe Asn Asp Val Ile Val Gly Ser Pro Leu Glu 535 540 545 550	1785
AAT CAG AAT TCT GGA GCT GTA TAC ATT TAC AAT GGT CAT CAG GGC ACT Asn Gln Asn Ser Gly Ala Val Tyr Ile Tyr Asn Gly His Gln Gly Thr 555 560 565	1833
ATC CGC ACA AAG TAT TCC CAG AAA ATC TTG GGA TCC GAT GGA GCC TTT Ile Arg Thr Lys Tyr Ser Gln Lys Ile Leu Gly Ser Asp Gly Ala Phe 570 576 580	1881
AGG AGC CAT CTC CAG TAC TTT GGG AGG TCC TTG GAT GGC TAT GGA GAT Arg Ser His Leu Gln Tyr Phe Gly Arg Ser Leu Asp Gly Tyr Gly Asp 585 590 595	1929
TTA AAT GGG GAT TCC ATC ACC GAT GTG TCT ATT GGT GCC TTT GGA CAA Leu Asn Gly Asp Ser Ile Thr Asp Val Ser Ile Gly Ala Phe Gly Gln 600 605 610	1977
GTG GTT CAA CTC TGG TCA CAA AGT ATT GCT GAT GTA GCT ATA GAA GCT Val Val Gln Leu Trp Ser Gln Ser Ile Ala Asp Val Ala Ile Glu Ala 615 620 625 630	2025
TCA TTC ACA CCA GAA AAA ATC ACT TTG GTC AAC AAG AAT GCT CAG ATA Ser Phe Thr Pro Glu Lys Ile Thr Leu Val Asn Lys Asn Ala Gln Ile 635 640 645	2073
ATT CTC AAA CTC TGC TTC AGT GCA AAG TTC AGA CCT ACT AAG CAA AAC Ile Leu Lys Leu Cys Phe Ser Ala Lys Phe Arg Pro Thr Lys Gln Asn 650 655 660	2121
AAT CAA GTG GCC ATT GTA TAT AAC ATC ACA CTT GAT GCA GAT GGA TTT Asn Gln Val Ala Ile Val Tyr Asn Ile Thr Leu Asp Ala Asp Gly Phe 665 670 675	2169
TCA TCC AGA GTA ACC TCC AGG GGG TTA TTT AAA GAA AAC AAT GAA AGG Ser Ser Arg Val Thr Ser Arg Gly Leu Phe Lys Glu Asn Asn Glu Arg 680 685 690	2217
TGC CTG CAG AAG AAT ATG GTA GTA AAT CAA GCA CAG AGT TGC CCC GAG Cys Leu Gln Lys Asn Met Val Val Asn Gln Ala Gln Ser Cys Pro Glu 695 700 715 720	2265

CAC ATC ATT TAT ATA CAG GAG CCC TCT GAT GTT GTC AAC TCT TTG GAT His Ile Ile Tyr Ile Gln Glu Pro Ser Asp Val Val Asn Ser Leu Asp 725 730 735	2313
TTG CGT GTG GAC ATC AGT CTG GAA AAC CCT GGC ACT AGC CCT GCC CTT Leu Arg Val Asp Ile Ser Leu Glu Asn Pro Gly Thr Ser Pro Ala Leu 740 745 750	2361
GAA GCC TAT TCT GAG ACT GCC AAG GTC TTC AGT ATT CCT TTC CAC AAA Glu Ala Tyr Ser Glu Thr Ala Lys Val Phe Ser Ile Pro Phe His Lys 755 760 765	2409
GAC TGT GGT GAG GAT GGA CTT TGC ATT TCT GAT CTA GTC CTA GAT GTC Asp Cys Gly Glu Asp Gly Leu Cys Ile Ser Asp Leu Val Leu Asp Val 760 765 770	2457
CGA CAA ATA CCA GCT GCT CAA GAA CAA CCC TTT ATT GTC AGC AAC CAA Arg Gln Ile Pro Ala Ala Gln Glu Gln Pro Phe Ile Val Ser Asn Gln 775 780 785 790	2505
AAC AAA AGG TTA ACA TTT TCA GTA ACA CTG AAA AAT AAA AGG GAA AGT Asn Lys Arg Leu Thr Phe Ser Val Thr Leu Lys Asn Lys Arg Glu Ser 795 800 805	2553
GCA TAC AAC ACT GGA ATT GTT GAT TTT TCA GAA AAC TTG TTT TTT Ala Tyr Asn Thr Gly Ile Val Val Asp Phe Ser Glu Asn Leu Phe Phe 810 815 820	2601
GCA TCA TTC TCC CTA CCG GTT GAT GGG ACA GAA GTA ACA TGC CAG GTG Ala Ser Phe Ser Leu Pro Val Asp Gly Thr Glu Val Thr Cys Gln Val 825 830 835	2649
GCT GCA TCT CAG AAG TCT GTT GCC TGC GAT GTA GGC TAC CCT GCT TTA Ala Ala Ser Gln Lys Ser Val Ala Cys Asp Val Gly Tyr Pro Ala Leu 840 845 850	2697
AAG AGA GAA CAA CAG GTG ACT TTT ACT ATT AAC TTT GAC TTC AAT CTT Lys Arg Glu Gln Gln Val Thr Phe Thr Ile Asn Phe Asp Phe Asn Leu 855 860 865 870	2745
CAA AAC CTT CAG AAT CAG GCG TCT CTC AGT TTC CAA GCC TTA AGT GAA Gln Asn Leu Gln Asn Gln Ala Ser Leu Ser Phe Gln Ala Leu Ser Glu 875 880 885	2793
AGC CAA GAA AAC AAG GCT GAT AAT TTG GTC AAC CTC AAA ATT CCT Ser Gln Glu Glu Asn Lys Ala Asp Asn Leu Val Asn Leu Lys Ile Pro 890 895 900	2841
CTC CTG TAT GAT GCT GAA ATT CAC TTA ACA AGA TCT ACC AAC ATA AAT Leu Leu Tyr Asp Ala Glu Ile His Leu Thr Arg Ser Thr Asn Ile Asn 905 910 915	2889
TTT TAT GAA ATC TCT TCG GAT GGG AAT GTT CCT TCA ATC GTG CAC AGT Phe Tyr Glu Ile Ser Ser Asp Gly Asn Val Pro Ser Ile Val His Ser 920 925 930	2937

TTT GAA GAT GTT GGT CCA AAA TTC ATC TIC TCC CTG AAG GTA ACA ACA Phe Glu Asp Val Gly Pro Lys Phe Ile Phe Ser Leu Lys Val Thr Thr 935                    940                    945                    950	2985
GGA AGT GTT CCA GTA AGC ATG GCA ACT GTA ATC ATC CAC ATC CCT CAG Gly Ser Val Pro Val Ser Met Ala Thr Val Ile Ile His Ile Pro Gln 955                    960                    965	3033
TAT ACC AAA GAA AAG AAC CCA CTG ATG TAC CTA ACT GGG GTG CAA ACA Tyr Thr Lys Glu Lys Asn Pro Leu Met Tyr Leu Thr Gly Val Gln Thr 970                    975                    980	3081
GAC AAG GCT GGT GAC ATC AGT TGT AAT GCA GAT ATC AAT CCA CTG AAA Asp Lys Ala Gly Asp Ile Ser Cys Asn Ala Asp Ile Asn Pro Leu Lys 985                    990                    995	3129
ATA GGA CAA ACA TCT TCT GTA TCT TTC AAA AGT GAA AAT TTC AGG Ile Gly Gln Thr Ser Ser Val Ser Phe Lys Ser Glu Asn Phe Arg 1000                    1005                    1010	3177
CAC ACC AAA GAA TTG AAC TGC AGA ACT GCT TCC TGT AGT AAT GTT ACC His Thr Lys Glu Leu Asn Cys Arg Thr Ala Ser Cys Ser Asn Val Thr 1015                    1020                    1025                    1030	3225
TGC TGG TTG AAA GAC GTT CAC ATG AAA GGA GAA TAC TTT GTT AAT GTG Cys Trp Leu Lys Asp Val His Met Lys Gly Glu Tyr Phe Val Asn Val 1035                    1040                    1045	3273
ACT ACC AGA ATT TGG AAC GGG ACT TTC GCA TCA TCA ACG TTC CAG ACA Thr Thr Arg Ile Trp Asn Gly Thr Phe Ala Ser Ser Thr Phe Gln Thr 1050                    1055                    1060	3321
GTA CAG CTA ACG GCA GCT GCA GAA ATC AAC ACC TAT AAC CCT GAG ATA Val Gln Leu Thr Ala Ala Glu Ile Asn Thr Tyr Asn Pro Glu Ile 1065                    1070                    1075	3369
TAT GTG ATT GAA GAT AAC ACT GTT ACG ATT CCC CTG ATG ATA ATG AAA Tyr Val Ile Glu Asp Asn Thr Val Thr Ile Pro Leu Met Ile Met Lys 1080                    1085                    1090	3417
CCT GAT GAG AAA GCC GAA GTA CCA ACA GGA GTT ATA ATA GGA AGT ATA Pro Asp Glu Lys Ala Glu Val Pro Thr Gly Val Ile Ile Gly Ser Ile 1095                    1100                    1105                    1110	3465
ATT GCT GGA ATC CTT TTG CTG TTA GCT CTG GTT GCA ATT TTA TGG AAG Ile Ala Gly Ile Leu Leu Leu Ala Leu Val Ala Ile Leu Trp Lys 1115                    1120                    1125	3513
CTC GGC TTC TTC AAA AGA AAA TAT GAA AAG ATG ACC AAA AAT CCA GAT Leu Gly Phe Lys Arg Lys Tyr Glu Lys Met Thr Lys Asn Pro Asp 1130                    1135                    1140	3561
GAG ATT GAT GAG ACC ACA GAG CTC AGT AGC TGAACCAGCA GACCTACCTG CAGT Glu Ile Asp Glu Thr Thr Glu Leu Ser Ser 1145                    1150	3615

GGGAACCGGC AGCATCCAG CCAGGGTTG CTGTTTGGT GCATGGATT CTTTTAAAT	3675
CCCATATTT TTTTATCATG TCGTAGGTAA ACTAACCTGG TATTTTAAGA GAAAAGTC	3735
GGTCAGTTG GATGAAGAAA TTGTGGGGG TGGGGGAGGT GCGGGGGCA GGTAGGGAAA	3795
TAATAGGGAA AATAACCTATT TTATATGATG GGGGAAAAAA AGTAATCTTT AAACCTGGCTG	3855
GCCCCAGAGTT TACATTCTAA TTTGCATTGT GTCAGAAACA TGAAATGCTT CCAAGCATGA	3915
CAACTTTAA AGAAAAATAT GATACTCTCA GATTTAAGG GGGAAAATC TTCTCTTAA	3975
AATATTTGTC TTTAACACGC AACTACAGAA GTGGAAGTGC TTGATATGTA AGTACTTCCA	4035
CTTGTGTATA TTTTAATGAA TATTGATGTT AACAAAGAGGG GAAAACAAAAA CACAGGTTT	4095
TTCAATTAT GCTGCTCATC CAAAGTTGCC ACAGATGATA CTTCCAAGTG ATAATTTAT	4155
TTATAAACTA GGTAAAATTG TTGTTGGTT CCTTTTATAC CACGGCTGCC CCTTCCACAC	4215
CCCATCTTGC TCTAATGATC AAAACATGCT TGAATAACTG AGCTTAGAGT ATACCTCTA	4275
TATGTCCATT TAAGTTAGGA GAGGGGGCGA TATAGAGACT AAGGCACAAA ATTTTGTAA	4335
AAACTCAGAA TATAACATTT ATGTAACATC CCATCTGCTA GAAGCCCATC CTGTGCCAGA	4395
GGAGGAAAA GGAGGAAATT TCCTTCTCT TTTAGGAGC ACAACAGTTC TCTTCTAGGA	4455
TTTGTGGC TGACTGGCAG TAACCTAGTG AATTTTGAA AGATGAGTAA TTTCTTGGC	4515
AACCTTCTC CTCCCTTACT GAACCACTCT CCCACCTCTT GGTTGACCA TTATTATAGA	4575
AGCCCTCTAC AGCCTGACTT TCTCTCCAGC GGTCCAAAGT TATCCCTC TTTACCCCTC	4635
ATCCAAAGTT CCCACTCTT CAGGACAGCT GCTGTGCATT AGATATTAGG GGGGAAAGTC	4695
ATCTGTTAA TTTACACACT TGATGAATT ACTGTATATA AACTCTTAA TTTCAGGGAG	4755
CTATTTCTAT TTAGTGCTAA ACAAGTAAGA AAAATAAGCT AGAGTGAATT TCTAAATGTT	4815
GGAATGTTAT GGGATGTTAA CAATGTAAG TAAAACACTC TCAGGATTT ACCAGAAGTT	4875
ACAGATGAGG CACTGGAAAC CACCACAAA TTAGCAGGTG CACCTCTGT GGCTGTCTTG	4935
TTTCTGAAGT ACTTTTCTT CCACAAGAGT GAATTGACC TAGGCAAGTT TGTTCAAAG	4995
GTAGATCTG AGATGATTG GTCAGATTGG GATAAGGCC AGCAATCTGC ATTTTAACAA	5055
GCACCCAGT CACTGGATG CAGATGGACC ACACITGAG AAACACCACC CATTTCTACT	5115
TTTGTGACCT TATTTCTCT GTTCCTGAGC CCCCACATT TCTAGGAGAA ACTTAGATTA	5175
AAATTCCACAG ACACATACA TCTAAAGCTT TGACAAGTCC TTGACCTCTA TAAACTTCAG	5235
AGTCCTCATT ATAAAATGGG AAGACTGAGC TGGAGTTCAAG CAGTGATGCT TTTTAGTTT	5295
AAAAGTCTAT GATCTGATCT GGACTTCCTA TAATACAAAT ACACAATCCT CCAAGAAATT	5355
GACTTGGAAA AGGAATTC	5373

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1181 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE: internal
- (vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Gly Pro Glu Arg Thr Gly Ala Ala Pro Leu Pro Leu Leu Val  
 -29            -25            -20            -15

Leu Ala Leu Ser Gln Gly Ile Leu Asn Cys Cys Leu Ala Tyr Asn Val  
 -10            -5            1

Gly Leu Pro Glu Ala Lys Ile Phe Ser Gly Pro Ser Ser Glu Gln Phe  
 5            10            15

Gly Tyr Ala Val Gln Gln Phe Ile Asn Pro Lys Gly Asn Trp Leu Leu  
20 25 30 35

Val Gly Ser Pro Trp Ser Gly Phe Pro Glu Asn Arg Met Gly Asp Val  
40 45 50

Tyr Lys Cys Pro Val Asp Leu Ser Thr Ala Thr Cys Glu Lys Leu Asn  
55 60 65

Leu Gln Thr Ser Thr Ser Ile Pro Asn Val Thr Glu Met Lys Thr Asn  
70 75 80

Met Ser Leu Gly Leu Ile Leu Thr Arg Asn Met Gly Thr Gly Gly Phe  
85 90 95

Leu Thr Cys Gly Pro Leu Trp Ala Gln Gln Cys Gly Asn Gln Tyr Tyr  
100 105 110 115

Thr Thr Gly Val Cys Ser Asp Ile Ser Pro Asp Phe Gln Leu Ser Ala  
120 125 130

Ser Phe Ser Pro Ala Thr Gln Pro Cys Pro Ser Leu Ile Asp Val Val  
135 140 145

Val Val Cys Asp Glu Ser Asn Ser Ile Tyr Pro Trp Asp Ala Val Lys  
150 155 160

Asn Phe Leu Glu Lys Phe Val Gln Gly Leu Asp Ile Gly Pro Thr Lys  
165 170 175

Thr Gln Val Gly Leu Ile Gln Tyr Ala Asn Asn Pro Arg Val Val Phe  
180 185 190 195

Asn Leu Asn Thr Tyr Lys Thr Lys Glu Glu Met Ile Val Ala Thr Ser  
200 205 210

Gln Thr Ser Gln Tyr Gly Asp Leu Thr Asn Thr Phe Gly Ala Ile  
215 220 225

Gln Tyr Ala Arg Lys Tyr Ala Tyr Ser Ala Ala Ser Gly Gly Arg Arg  
230 235 240

Ser Ala Thr Lys Val Met Val Val Thr Asp Gly Glu Ser His Asp  
245 250 255

Gly Ser Met Leu Lys Ala Val Ile Asp Gln Cys Asn His Asp Asn Ile  
260 265 270 275

Leu Arg Phe Gly Ile Ala Val Leu Gly Tyr Leu Asn Arg Asn Ala Leu  
280 285 290

Asp Thr Lys Asn Leu Ile Lys Glu Ile Lys Ala Ile Ala Ser Ile Pro  
295 300 305

Thr Glu Arg Tyr Phe Phe Asn Val Ser Asp Glu Ala Ala Leu Leu Glu  
310 315 320

Lys Ala Gly Thr Leu Gly Glu Gln Ile Phe Ser Ile Glu Gly Thr Val  
 325                    330                    335

Gln Gly Gly Asp Asn Phe Gln Met Glu Met Ser Gln Val Gly Phe Ser  
 340                    345                    350                    355

Ala Asp Tyr Ser Ser Gln Asn Asp Ile Leu Met Leu Gly Ala Val Gly  
 360                    365                    370

Ala Phe Gly Trp Ser Gly Thr Ile Val Gln Lys Thr Ser His Gly His  
 375                    380                    385

Leu Ile Phe Pro Lys Gln Ala Phe Asp Gln Ile Leu Gln Asp Arg Asn  
 390                    395                    400

His Ser Ser Tyr Leu Gly Tyr Ser Val Ala Ala Ile Ser Thr Gly Glu  
 405                    410                    415

Ser Thr His Phe Val Ala Gly Ala Pro Arg Ala Asn Tyr Thr Gly Gln  
 420                    425                    430                    435

Ile Val Leu Tyr Ser Val Asn Glu Asn Gly Asn Ile Thr Val Ile Gln  
 440                    445                    450

Ala His Arg Gly Asp Gln Ile Gly Ser Tyr Phe Gly Ser Val Leu Cys  
 455                    460                    465

Ser Val Asp Val Asp Lys Asp Thr Ile Thr Asp Val Leu Leu Val Gly  
 470                    475                    480

Ala Pro Met Tyr Met Ser Asp Leu Lys Lys Glu Gly Arg Val Tyr  
 485                    490                    495

Leu Phe Thr Ile Lys Lys Gly Ile Leu Gly Gln His Gln Phe Leu Glu  
 500                    505                    510                    515

Gly Pro Glu Gly Ile Glu Asn Thr Arg Phe Gly Ser Ala Ile Ala Ala  
 520                    525                    530

Leu Ser Asp Ile Asn Met Asp Gly Phe Asn Asp Val Ile Val Gly Ser  
 535                    540                    545

Pro Leu Glu Asn Gln Asn Ser Gly Ala Val Tyr Ile Tyr Asn Gly His  
 550                    555                    560

Gln Gly Thr Ile Arg Thr Lys Tyr Ser Gln Lys Ile Leu Gly Ser Asp  
 565                    570                    575

Gly Ala Phe Arg Ser His Leu Gln Tyr Phe Gly Arg Ser Leu Asp Gly  
 580                    585                    590                    595

Tyr Gly Asp Leu Asn Gly Asp Ser Ile Thr Asp Val Ser Ile Gly Ala  
 600                    605                    610

Phe Gly Gln Val Val Gln Leu Trp Ser Gln Ser Ile Ala Asp Val Ala  
 615                    620                    625

Ile Glu Ala Ser Phe Thr Pro Glu Lys Ile Thr Leu Val Asn Lys Asn  
630 635 640

Ala Gln Ile Ile Leu Lys Leu Cys Phe Ser Ala Lys Phe Arg Pro Thr  
645 650 655

Lys Gln Asn Asn Gln Val Ala Ile Val Tyr Asn Ile Thr Leu Asp Ala  
660 665 670 675

Asp Gly Phe Ser Ser Arg Val Thr Ser Arg Gly Leu Phe Lys Glu Asn  
680 685 690

Asn Glu Arg Cys Leu Gln Lys Asn Met Val Val Asn Gln Ala Gln Ser  
695 700 705

Cys Pro Glu His Ile Ile Tyr Ile Gln Glu Pro Ser Asp Val Val Asn  
710 715 720

Ser Leu Asp Leu Arg Val Asp Ile Ser Leu Glu Asn Pro Gly Thr Ser  
725 730 735

Pro Ala Leu Glu Ala Tyr Ser Glu Thr Ala Lys Val Phe Ser Ile Pro  
740 745 750 755

Phe His Lys Asp Cys Gly Glu Asp Gly Leu Cys Ile Ser Asp Leu Val  
760 765 770

Leu Asp Val Arg Gln Ile Pro Ala Ala Gln Glu Gln Pro Phe Ile Val  
775 780 785

Ser Asn Gln Asn Lys Arg Leu Thr Phe Ser Val Thr Leu Lys Asn Lys  
790 795 800

Arg Glu Ser Ala Tyr Asn Thr Gly Ile Val Val Asp Phe Ser Glu Asn  
805 810 815

Leu Phe Phe Ala Ser Phe Ser Leu Pro Val Asp Gly Thr Glu Val Thr  
820 825 830 835

Cys Gln Val Ala Ala Ser Gln Lys Ser Val Ala Cys Asp Val Gly Tyr  
840 845 850

Pro Ala Leu Lys Arg Glu Gln Gln Val Thr Phe Thr Ile Asn Phe Asp  
855 860 865

Phe Asn Leu Gln Asn Leu Gln Asn Gln Ala Ser Leu Ser Phe Gln Ala  
870 875 880

Leu Ser Glu Ser Gln Glu Glu Asn Lys Ala Asp Asn Leu Val Asn Leu  
885 890 895

Lys Ile Pro Leu Leu Tyr Asp Ala Glu Ile His Leu Thr Arg Ser Thr  
900 905 910 915

Asn Ile Asn Phe Tyr Glu Ile Ser Ser Asp Gly Asn Val Pro Ser Ile  
920 925 930

Val His Ser Phe Glu Asp Val Gly Pro Lys Phe Ile Phe Ser Leu Lys  
935 940 945

Val Thr Thr Gly Ser Val Pro Val Ser Met Ala Thr Val Ile Ile His  
950 955 960

Ile Pro Gln Tyr Thr Lys Glu Lys Asn Pro Leu Met Tyr Leu Thr Gly  
965 970 975

Val Gln Thr Asp Lys Ala Gly Asp Ile Ser Cys Asn Ala Asp Ile Asn  
980 985 990 995

Pro Leu Lys Ile Gly Gln Thr Ser Ser Val Ser Phe Lys Ser Glu  
1000 1005 1010

Asn Phe Arg His Thr Lys Glu Leu Asn Cys Arg Thr Ala Ser Cys Ser  
1015 1020 1025

Asn Val Thr Cys Trp Leu Lys Asp Val His Met Lys Gly Glu Tyr Phe  
1030 1035 1040

Val Asn Val Thr Thr Arg Ile Trp Asn Gly Thr Phe Ala Ser Ser Thr  
1045 1050 1055

Phe Gln Thr Val Gln Leu Thr Ala Ala Ala Glu Ile Asn Thr Tyr Asn  
1060 1065 1070 1075

Pro Glu Ile Tyr Val Ile Glu Asp Asn Thr Val Thr Ile Pro Leu Met  
1080 1085 1090

Ile Met Lys Pro Asp Glu Lys Ala Glu Val Pro Thr Gly Val Ile Ile  
1095 1100 1105

Gly Ser Ile Ile Ala Gly Ile Leu Leu Leu Ala Leu Val Ala Ile  
1110 1115 1120

Leu Trp Lys Leu Gly Phe Phe Lys Arg Lys Tyr Glu Lys Met Thr Lys  
1125 1130 1135

Asn Pro Asp Glu Ile Asp Glu Thr Thr Glu Leu Ser Ser  
1140 1145 1150

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCAGAGTCAC TCTCACAGAG

20

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CACAGCGTAC ACGTACACC

19

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CACTTATAGA CATCTCCAG

19

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE:

## (vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CATCCATGTT GATGTCTG

18

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE:

## (vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CATGTGATTG ACCGTCAG

18

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTISENSE: NO

## (v) FRAGMENT TYPE:

## (vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCATATTGAA TTGCTCCGAA TGTG

24

## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGCGTATGCA CAACGCA

17

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCGACAGCTG ACCAGTCAGC A

21

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CACTCCTCCA CAGCTCCT

18

## (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 15 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
  
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:
  
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ACATGTACTC ACTGG

15

## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
  
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:
  
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTCACATGTG GTCTCTG

18

## (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
  
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:
  
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GTCCTGTTGA CCTATCCACT GC

22

## WE CLAIM:

1. A method to identify a mammal having or at risk for developing glomerulopathy comprising the steps of:
  - 5       analyzing a tissue sample from a mammal known to contain cells expressing integrin RNA or protein for integrin subunit expression; and  
            comparing integrin subunit expression in the sample with a control tissue sample, wherein altered integrin subunit expression is correlated with glomerulopathy.
  - 10      2. The method of Claim 1, wherein the mammal is a human.
  3. The method of Claim 1, wherein the tissue sample is a kidney biopsy.
  4. The method of Claim 1, wherein the tissue sample is blood.
  - 15      5. The method of Claim 4, wherein the blood sample contains polymorphonuclear cells or monocytes.
  6. The method of Claim 1, wherein the tissue sample is a skin biopsy.
  - 20      7. The method of Claim 1, wherein said analysis comprises *in situ* hybridization.
  8. The method of Claim 7, wherein said *in situ* hybridization comprises PCR enhanced *in situ* hybridization.
  - 25      9. The method of Claim 1, wherein said analyzing comprises isolating RNA from the sample.
  - 30      10. The method of Claim 1, wherein said analyzing comprises performing PCR, detecting amplified fragments from an integrin subunit and comparing the amount of amplified fragments to the amount of amplified fragments obtained from the control.

11. The method of claim 1, wherein the integrin subunit is an alpha integrin subunit.
12. The method of Claim 11, wherein the  $\alpha$  integrin subunit is  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ , or  $\alpha 5$  integrin subunit.  
5
13. The method of claim 12, wherein the  $\alpha$  integrin subunit is  $\alpha 1$  or  $\alpha 2$  integrin subunit.
14. The method of claim 1, wherein a decrease in  $\alpha 1$  integrin subunit in the tissue sample as compared with control tissue is correlated with nephropathy.  
10
15. The method of claim 1, wherein an increase in  $\alpha 2$   $\alpha 3$ ,  $\alpha 5$ , or  $\beta 1$  integrin subunit in the tissue sample as compared with control tissue is correlated with nephropathy.
16. The method of claim 1, wherein an increase in  $\alpha 2$  and a decrease in  $\alpha 1$  integrin subunit in the tissue sample as compared with control tissue is correlated with nephropathy.  
15
17. The method of Claim 7, wherein a nucleic acid probe is used to detect integrin, and the probe comprises a 3.9kb fragment of  $\alpha 1$  from the 5' end to nucleotide 3900.  
20
18. The method of Claim 7, wherein a nucleic acid probe is used to detect integrin, and the probe comprises a 1.8kb fragment of  $\alpha 2$  from 5' end through the EcoRI site at nucleotide 1800.  
25
19. The method of Claim 1, wherein said analyzing comprises incubating the sample with an anti-integrin subunit antibody.
20. The method of Claim 1, wherein the nondiabetic control sample is from a mammal with no history of hypertension.  
30

21. The method of Claim 1, wherein an increase of about 25% - 100% in the level of  $\alpha_2$  integrin subunit expression in the sample tissue as compared with the control is correlated with nephropathy.
- 5 22. The method of Claim 1, wherein a decrease of about 25% - 100% in the level of  $\alpha_1$  integrin subunit expression in the sample tissue as compared with the control is correlated with nephropathy.
- 10 23. A method to identify a mammal having or at risk for developing glomerulopathy comprising the steps of:  
analyzing a tissue sample from a mammal known to contain cells expressing integrin protein for  $\alpha_1$  and  $\alpha_2$  integrin subunit expression as compared with a control tissue sample; and  
correlating a decreased level of  $\alpha_1$  integrin subunit expression and/or an increased level of  $\alpha_2$  integrin subunit expression in the sample tissue as compared to the control with nephropathy.
- 15 24. A method to identify a mammal with diabetes who has or is at risk for developing secondary pathological changes associated with diabetes comprising the steps of:  
analyzing a tissue sample from a mammal known to contain cells expressing integrin protein for integrin subunit expression; and  
correlating alterations in the level of expression of least one integrin subunit as compared with a control tissue sample with the presence of or the risk for developing secondary pathological changes associated with diabetes.
- 20 25. The method of claim 25, wherein said integrin subunit is  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_5$ , or  $\beta_1$ .
- 25 26. The method of claim 25, wherein said integrin subunit is  $\alpha_1$  or  $\alpha_2$ .

## PATENT COOPERATION TREATY

PCT

PTO/REC'D 21 JAN 1998  
09/000004

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>600.314W001</b>	<b>FOR FURTHER ACTION</b>	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. <b>PCT/US 96/ 12067</b>	International filing date ( <i>day/month/year</i> ) <b>19/07/1996</b>	(Earliest) Priority Date ( <i>day/month/year</i> ) <b>21/07/1995</b>
Applicant <b>REGENTS OF THE UNIVERSITY OF MINNESOTA et al.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of **4** sheets.

It is also accompanied by a copy of each prior art document cited in this report.

1.  Certain claims were found unsearchable (see Box I).
  2.  Unity of invention is lacking (see Box II).
  3.  The international application contains disclosure of a nucleotide and/or amino acid sequence listing and the international search was carried out on the basis of the sequence listing
    - filed with the international application.
    - furnished by the applicant separately from the international application,
      - but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
    - Transcribed by this Authority
  4. With regard to the title,  the text is approved as submitted by the applicant.
    - the text has been established by this Authority to read as follows:
  5. With regard to the abstract,
    - the text is approved as submitted by the applicant.
    - the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.
  6. The figure of the drawings to be published with the abstract is:
    - Figure No. \_\_\_\_\_  as suggested by the applicant.
    - because the applicant failed to suggest a figure.
    - because this figure better characterizes the invention.
- None of the figures.

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 96/12067

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12Q1/68 C07K14/705 C07K16/28 G01N33/566

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NEPHRON (1992), 62(4), 382-8, XP000611529 BARALDI ET AL.: "Very late activation-3 integrin is the dominant .beta.1-integrin on the glomerular capillary wall: an immunofluorescence study in nephrotic syndrome" see the whole document ---	1-30
X	LABORATORY INVESTIGATION, vol. 72, no. 3, March 1995, pages 367-375, XP000611274 MENDRICK ET AL.: "Glomerular epithelial and mesangial cells differentially modulate the binding specificities of VLA-1 and VLA-2" cited in the application see page 372, right-hand column ---	1-30 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

2

Date of the actual completion of the international search Date of mailing of the international search report

28 November 1996

06.12.96

## Name and mailing address of the ISA

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## Authorized officer

Molina Galan, E

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/12067

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. AM. SOC. NEPHROLOGY, vol. 5, no. 3, 1994, page 966 XP002019387 KYU JIN ET AL.: "Skin fibroblast integrin expression in IDDM" cited in the application see abstract ---	1-30
X	AMERICAN JOURNAL OF KIDNEY DISEASES, (1995 MAY) 25 (5) 680-8, XP000611528 SHIKATA K; MAKINO H; MORIOKA S; KASHITANI T; HIRATA K; OTA Z; WADA: "Distribution of extracellular matrix receptors in various forms of glomerulonephritis." see the whole document ---	1-6,11, 12,19, 20,25,27
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1993 OCT 15) 90 (20) 9640-4, XP002019388 ROTH ET AL.: "Integrin overexpression induced by high glucose and by human diabetes: potential pathway to cell dysfunction in diabetic microangiopathy." see the whole document ---	1-6,11, 12,19, 20,25,27
X	INVESTIGATIVE OPHTHALMOLOGY AND VISUAL SCIENCE, vol. 35, no. 9, August 1994, pages 3475-3485, XP000611525 ROBBINS ET AL.: "Immunolocalisation of integrins in proliferative retinal membranes" See discussion see abstract ---	24
A	PCR METHODS AND APPLICATIONS, vol. 2, no. 2, 1 November 1992, pages 117-123, XP000472828 NUOVO G J ET AL: "IN SITU LOCALIZATION OF PCR-AMPLIFIED HUMAN AND VIRAL CDNAS" see the whole document ---	7-10
A	J. CELL BIOL., vol. 111, August 1990, pages 709-720, XP000611279 IGNATIUS ET AL.: "Molecular cloning of the rat integrin alpha 1 subunit" cited in the application see figure 2 ---	17
2	-/-	

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/12067

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. CELL BIOL., vol. 109, July 1989, pages 397-407, XP000611310 TAKADA ET AL.: "The primary structure of the VLA-2/collagen receptor alpha 2 subunit" cited in the application see figure 2 ---	18
A	FEBS LETTERS, vol. 332, no. 3, October 1993, AMSTERDAM NL, pages 263-267, XP002019390 ROZZO ET AL.: "Modulation of integrin heterodimers during human neuroblastoma cell differentiation" cited in the application ---	
A	JOURNAL OF BIOLOGICAL CHEMISTRY , vol. 268, no. 4, February 1993, MD US, pages 2989-2996, XP002019389 BRIESEWITZ ET AL.: "Expression of native and truncated forms of the human Integrin alpha-1 subunit" cited in the application ---	
P,X	CELL ADHESION AND COMMUNICATION, (1995 AUG) 3 (3) 187-200, XP000611503 SETTY S ; ANDERSON S S; WAYNER E A; KIM Y; CLEGG D O;; "Glucose-induced alteration of integrin expression and function in cultured human mesangial cells" see the whole document ---	1-30
P,X	ANNUAL MEETING OF THE AMERICAN SOCIETY OF NEPHROLOGY, SAN DIEGO, CALIFORNIA, USA, NOVEMBER 5-8, 1995. JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY 6 (3). 1995. 911., XP002019391 SETTY S ; WU K; MAUER M; KIM Y; TSILIBARY E C: "Altered mesangial expression of integrin genes in response to elevated glucose and experimental diabetes " see abstract -----	1-30
2		

## PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

REC'D 29 OCT. 1997

PCT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference  600.314W001	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No.  PCT/US 96/ 12067	International filing date (day/month/year)  19/07/1996	Priority date (day/month/year)  21/07/1995
International Patent Classification (IPC) or national classification and IPC  C12Q1/68		
Applicant  REGENTS OF THE UNIVERSITY OF MINNESOTA et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

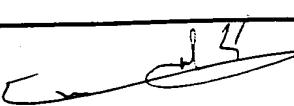
2. This REPORT consists of a total of 6 sheets, including this cover sheet.

This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consists of a total of \_\_\_\_\_ sheets.

3. This report contains indications and corresponding pages relating to the following items:

- I  Basis of the report
- II  Priority
- III  Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV  Lack of unity of invention
- V  Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI  Certain documents cited
- VII  Certain defects in the international application
- VIII  Certain observations on the international application

Date of submission of the demand  20/02/1997	Date of completion of this report  24.10.97
Name and mailing address of the IPEA  European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Netherlands Tel.: (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer  Molina Galan E.  Telephone No. 340 3560

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US96/12067

## I. Basis of the report

1. This report has been drawn up on the basis of (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*)

the international application as originally filed

the description, pages

as originally filed

pages

filed with the demand

pages

filed with the letter of

the claims, Nos.

as originally filed

Nos.

as amended under Article 19

Nos.

filed with the demand

Nos.

filed with the letter of

the drawings, sheets / fig.

as originally filed

sheets / fig.

filed with the demand

sheets / fig.

filed with the letter of

2. The amendments have resulted in the cancellation of:

the description, pages:

the claims, Nos.

the drawings, sheets / fig.

3.  This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2 (c)).

4. Additional observations, if necessary:

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US96/12067

**V. Reasoned statement under Article 35(2) with regard to novelty, inventiveness step or industrial applicability; citations and explanations supporting such statement****1. Statement**

Novelty	Claims	4, 5, 7, 8, 10, 14, 16-18, 20-22, 27 and 29	YES
	Claims	1-3, 6, 9, 11-13, 15, 19, 23-26, 28 and 30	NO
Inventive Step	Claims		YES
	Claims	1-30	NO
Industrial Applicability	Claims	1-30	YES
	Claims		NO

**2. Citations and Explanations****2.1 The following documents have been considered for the purposes of this report:**

D1:	Nephron,	vol. 62, 1992,	pp. 382-388;	Baraldi et al.
D2:	Lab. Invest.,	vol. 72, 3/1995	pp. 367-375;	Mendrick et al.
D3:	J. Am. Soc. Nephrology,	vol. 5, 1994,	page 966;	Kyu Jin et al.
D4:	Am. J. Kidney Dis.,	vol. 25, 5/1995,	pp. 680-688;	Shikata et al.
D5:	Proc. Natl. Acad. Sci.,	vol. 90, 1993,	pp. 9640-9644;	Roth et al.
D6:	Invest. Ophtal. Vis. Sci.,	vol. 35, 1994,	pp. 3475-3481;	Robbins et al.
D7:	FEBS,	vol. 332, 1993,	pp. 263-267;	Rozzo et al.
D8:	PCR Met. Appl.,	vol. 2, 1992,	pp. 117-123;	Nuovo et al.

**2.2 NOVELTY (Art. 33(2) PCT)**

2.2.1 D1 investigates the differential expression of  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$  and  $\alpha 6$  integrin subunits in normal subjects and patients affected by various glomerulopathies. Integrin expression is investigated on kidney samples (cf abstract) by immunological means (cf table 2). The conclusion is reached that VLA-3 (i.e.  $\alpha 3\beta 1$  integrin) is a sensitive marker for membranous nephropathy (cf page 387, right column, first full sentence). Although a moderate increase in  $\alpha 2$  expression is found in some specimens (cf page 384, right column), this is not taken as relevant and it is concluded that  $\alpha 2$ ,  $\alpha 5$  and  $\alpha 6$  expression does not significantly change in the glomerulopathies studied (cf last paragraph).

- 2.2.2 D2 observed up-regulation of VLA-2 (i.e.  $\alpha 2\beta 1$  integrin) expression in glomerular cells cultured under conditions resembling those seen in such cells during pathological states. A comparison is drawn with fibroblasts and metastatic melanoma cells which have increased synthesis of VLA-2 when placed in collagen gels. The conclusion is reached that although glomerular epithelial cells synthesize little or no VLA-2 in their normal environment *in situ*, they may increase their expression of this receptor when exposed to interstitial collagens secreted by endogenous or inflammatory cells as seen in glomerulopathies (cf page 372, right column).
- 2.2.3 D3 performs a similar analysis on  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$  and  $\beta 1$  integrin subunits. It correlates by Northern blotting increased expression of  $\alpha 3$  and  $\beta 1$  integrin mRNA in skin fibroblasts with IDDM patients with nephropathy (cf abstract).
- 2.2.4 D4 studies the distribution of integrins in various forms of Glomerulonephritis by immunological methods. It was found that Integrin  $\beta 1$  and  $\alpha v\beta 3$  were highly expressed in a kind of nephropathy. In a different nephropathy,  $\alpha 3\beta 1$  presented a decreased immunoreactivity (cf abstract).
- 2.2.5 D5 observes that high glucose concentrations induce increases in the mRNA levels of  $\alpha 3$ ,  $\alpha 5$  and  $\beta 1$  integrin subunits in isolates of human endothelial cells. An increase in  $\beta 1$  expression was also observed in retinal trypsin digests of patients with diabetic retinopathy (cf abstract).
- 2.2.6 D6 determines the expression pattern of integrins in human proliferative membranes (such as retinopathy vessels) and reaches the conclusion that integrins are involved in the pathogenesis of diabetes (cf page 3483, left column, last full paragraph).
- 2.2.7 The present application does not satisfy the criterion set forth in Article 33(2) PCT because the subject-matter of Claims 1-3, 6, 9, 11-13, 15, 19, 23-26, 28 and 30 is not new in respect of prior art as defined in the regulations (Rule 64(1)-(3) PCT).

### 2.3 INVENTIVE STEP (Art. 33(3) PCT)

- 2.3.1 Document D1 is considered to represent the most relevant state of the art and has been discussed in 2.2.1 above. Claim 14 differs only in that decrease of expression of  $\alpha 1$  integrin subunit, with respect to a control, is used as a marker to asses risk for developing glomerulopathy.

- 2.3.2 The problem to be solved by the present invention may therefore be regarded as providing alternative markers for the identification of mammals having, or being at risk for developing, glomerulopathy. The solution is the use of decreased  $\alpha 1$  expression as a marker.
- 2.3.3 This solution cannot however be considered as involving an inventive step (Article 33(3) PCT) because the person skilled in the art would be prompted to investigate the use of  $\alpha 1$  integrin subunit as such a marker seen the broadly documented fact (e.g. D1-D6) that other  $\alpha$  and  $\beta$  integrin subunits are directly correlated with (and used as markers for) different glomerulopathic conditions as well as other secondary changes associated with diabetes. The fact that  $\alpha 1$  integrin subunit presents a decreased instead of an increased expression in tissues affected by said pathologies would undoubtedly be established by routine experimentation (such as the one performed in D7) without involvement of any inventive skills.
- 2.3.4 Dependent Claims 4, 5, 7, 8, 10, 16-18, 20-22, 27 and 29 do not appear to contain any additional features which, in combination with the features of any claim to which they refer, involve an inventive step because they are either a mere choice of different possibilities available to the person skilled in the art (for instance the use of in situ PCR is known from D8) or already anticipated by the cited literature.
- 2.3.5 The present application does therefore not satisfy the criterion set forth in Article 33(3) PCT and the subject-matter of Claims 4, 5, 7, 8, 10, 14, 16-18, 20-22, 27 and 29 does not involve an inventive step (Rule 65(1)(2) PCT).

**INTERNATIONAL PRELIMINARY EXAMINATION REPORT**

International application No.

PCT/US96/12067

**VII. Certain defects in the international application**

The following defects in the form or contents of the international application have been noted:

Documents D1, D4-D6 and D8 have not been identified in the description nor has the relevant background art disclosed therein been discussed. The requirements of Rule 5.1(a)(ii) PCT are, thus, not fulfilled.